Long-term effects of LPS and high-fat diet on dopaminergic neurodegeneration

Andrea Stojakovic

A thesis submitted for the degree of
Doctor of Philosophy of
The Australian National University

December 2013

This work was conducted in the Department of Translational Medicine from October 2010 to December 2013, The John Curtin School of Medical Research, Canberra, Australia. This thesis is my original work except where indicated.

Andrea Stojakovic

Canberra, Australia

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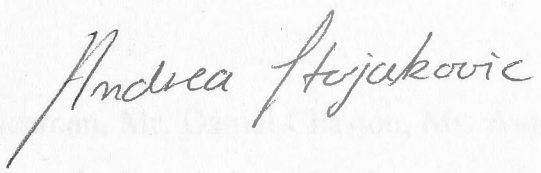


Acknowledgments

I would like to thank people that have helped me to complete this PhD project. First and foremost, I express my sincere gratitude to my supervisor Dr. Claudio A. Mastronardi on his guidance and scientific input in the project. Moreover, I would like to thank my co-supervisors and panel committee members: Prof. Ma-Li Wong M.D, Gilberto Paz-Filho M.D., Prof. Julio Licinio M.D., and Prof. Philip Board for their contribution and suggestions in this work. I extend my appreciation to Dr. Anna Cowan for her support and guidance in my PhD study.

I am grateful to Ms. Yeping Cai, Dr. Teresa Neeman, Mr. Daniel Chaston, Ms. Aulikki Koskinen, Ms. Anne Prins and Dr. Maria Staykova for their help and technical advice.

I thank my friends and colleges Ms. Suhyun Lee, Ms. Sooshin Choi, Mr. Antonio Inserra and Mr. Ameet K. Mishra on their help and support.

Lastly, I owe my deepest gratitude to my parents, on their immense emotional support and encouragement.

Abstract

Mounting evidence suggests that inflammation is involved in the etiology of neurodegenerative disorders such as Parkinson`s disease. It has been reported that the long-term effect of five doses of lipopolysaccharide from Gram-negative bacteria in mice caused activation of microglia and dopaminergic neurodegeneration that was reflected as decreased locomotor activity. In the current study, it is hypothesized that IL-1β, a potent proinflammatory cytokine synthesized and released by glia cells, could be mediating the LPS-induced dopaminergic neurodegeneration and that the chronic low-grade inflammatory state caused by diet-induced obesity would exacerbate LPS-induced neurodegeneration.

To ascertain the participation of IL-1β, three strains of mice were employed: a control [wild type (WT)], a strain lacking the endogenous antagonist of IL-1, namely IL-1 receptor antagonist (IL-1ra-/-), and a mouse strain deficient in caspase-1 (Casp-1-/-), a protease that cleaves the immature IL-1β into the mature, biologically active form. Mice of the three genotypes were fed a regular diet (RD) and additionally WT mice were fed a high-fat diet (HFD). All mouse groups were challenged with intraperitoneal injections of either saline or LPS (5 mg/kg; one or five times on a monthly basis).

Significant genotype and diet effects were observed in behavioral, metabolic and inflammatory outcomes over a period of 9 to 15 months, whereas the effect of LPS was modest or undetectable.

Compared to WT mice, Casp-1-/- mice preserved their locomotor activities, while IL-1ra-/- mice showed a time-dependent decline in motor and coordinative abilities. Non-motor symptoms included age-related development of anxiety-like behavior in IL-1ra-/- mice. Impairment of cognitive function was observed in Casp-1-/- mice. Since insulin and leptin may modulate dopamine neurotransmission, plasma levels of these two hormones were assessed. Casp-1-/- mice had increased plasma insulin levels but were not glucose intolerant, whereas IL-1ra-/- mice were hypoinsulinemic, but insulin sensitive. Leptin levels were reduced in both genotypes (Casp-1-/- and IL-1ra-/-). Casp-1-/- mice had intact dopamine neurons and less activated microglia cells. Dopamine neurodegeneration was observed in IL-1ra-/- mice and accompanied by higher, but non-significant activation of microglia. The connection between peripheral and central

inflammation was assessed by plasma level of monocyte chemoattractant protein-1 (MCP-1). Plasma MCP-1 tended to be increased by repeated LPS injections in WT mice and surprisingly it was reduced by single and repeated LPS injections in both Casp-1-/- and IL-1ra-/- mice.

Significant differences in final outcomes were observed between the two diet-fed groups of mice. LPS treatments did not induce motor decline in RD and HFD-fed mice during nine months of experiments. However, HFD mice showed symptoms of akinesia, bradykinesia, dyskinesia and reduced coordinative abilities. Non-motor symptoms observed in HFD mice were anxiety-like behavior in novelty suppressed feeding test, cognitive and partial memory impairment. HFD mice displayed glucose intolerance, high levels of leptin and insulin. Dopamine neurons were not affected by HFD and significant decreases were induced by repeated LPS injections. Loss of neurons was not accompanied with increased activation of microglia cells and MCP-1.

Overall, the data shown here emphasized the importance of the IL-1 signaling pathway in dopaminergic neurodegeneration.

Publications and meeting presentations

Poster presentations

Chapter 3:

Andrea Stojakovic, Antonio Inserra, Julio Licinio, Ma-Li Wong and Gilberto Paz-Filho and Claudio Mastronardi. Role of the IL-1 pathway in the LPS-induced decrease of dopaminergic neurons. Australian National University – The John Curtin School of Medical Research, 2011.

Chapter 3:

Andrea Stojakovic, Antonio Inserra, Gilberto Paz-Filho, Julio Licinio, Ma-Li Wong and Claudio Mastronardi. The role of the IL-1 pathway in LPS-induced behaviours. 4th Iberoamerican Congress on Neuroimmunomodulation and the 1st Mexican Congress of Neuroimmunoendocrinology, 2013, Puebla, Mexico.

Manuscript preparation

Andrea Stojakovic, Gilberto Paz-Filho, Julio Licinio, Ma-Li Wong and Claudio Mastronardi. Possible role of the IL-1 pathway in dopaminergic neurodegeneration.

Abbreviations

ACTH	Adrenocorticotropic hormone
AGRP	Agouti-related protein
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AP-1	Activator protein-1
APc	Antigen-presenting cells
ASC	Caspase recruitment domain
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BDNF	Brain-derived neurotrophic factor
BH4	Tetrahydrobiopterin
BMC	Bone mineral content
BMD	Bone mineral density
BW	Body weight
CARD	Caspase activation and recruitment domain
CART	Cocaine- and amphetamine-regulated transcript
Casp-1	Caspase-1
CCR2	Chemokine (C-C motif) receptor 2
CD/TD	Center to total distance ratio
CNS	Central nervous system
COX-2	Cyclooxygenase-2
CRF	Corticotropin releasing factor
CRH	Corticotropin-releasing hormone
CVO`s	Circumventricular organs
DAMP	Danger-associated molecular patterns
DAT	Dopamine active transporter

dB	Decibel
DEXA	Dual-emission X-ray absorptiometry
DOPAC	3, 4 dihydroxyphenylacetic acid
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
EP1	Endogenous pyrogen 1
ER-α	Estrogen receptor-α
GABA	Gamma-aminobutyric acid
GH	Growth hormone
GHIH	Growth hormone-inhibiting hormone
GHRH	Growth hormone-releasing hormone
GLUT-4	Glucose transporter type 4
GP	Globus pallidus
H_2O_2	Hydrogen peroxide
HFD	High-fat diet
HFD-NI	Non- injected WT mice fed a high-fat diet
HPA	Hypothalamic-pituitary-adrenal axis
HVA	Homovanillic acid
i.c.v.	Intracerebroventricular
i.p.	Intraperitoneal
ICAM-1	Intercellular adhesion molecule-1
icIL-1ra	Intracellular form of interleukin-1 receptor antagonist
IFN-α	Interferon-α
IFN-γ	Interferon-γ
IKK	I kappa B kinase
IL	Interleukin
IL-18BP	Indentified soluble protein of IL-18

IL-1R1	Interleukin 1 receptor, type I
IL-1ra	Interleukin-1 receptor antagonist
IL-1RAcP	IL-1 receptor accessory protein
iNOS	Inducible nitric oxide synthase
IP-10	Interferon gamma-induced protein-10
IPGTT	Intraperitoneal glucose tolerance test
IRAK4	Interleukin-1 receptor associated kinase 4
IRS	Insulin receptor substrate
JAM-A	Junctional adhesion molecule-A
L-DOPA	L-3,4-dihydroxyphenylalanine
LFA-1	Lymphocyte functional antigen-1
LHA	Lateral hypothalamic area
LPS	Lipopolysaccharide
LRR	Leucine-rich repeat
LTD	Long-term depression
LTP	Long-term potentiation
LXRα	Liver X receptor-α
MAO	Monoamine oxidase
MAO-B	Monoamine oxidase B
MAPK	Mitogen-activated protein kinase
MC3/4 R`s	Melanocortin- 3/ 4 receptors
MCP-1	Monocyte chemoattractant protein-1
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1α
MMP-3	Matrix metalloproteinase-3
MMP-9	Matrix metalloproteinase-9
MPP (+)	1-methyl-4-phenylpyridinium

MPTP-1	Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MyD88	Adaptor myeloid differentiation factor (88)
n-6 PUFA	Omega-6 polyunsaturated fatty acids
NACHT	Central nucleotide-binding domain
NALP3	PYD domains-containing protein 3
NEO	Neomycin resistance gene
NF-κB	Nuclear transcription factor
NGF	Nerve growth factor
NLR	Nucleotide-binding oligomerization domain-like receptor
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
NO_3^-	Peroxynitrite
NPY	Neuropeptide Y
nSMase	Sphingomyelinase
O_2^-	Superoxide anion
P2X7	Purinergic receptors
PAMP`s	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PFA	Perifornical area
PGE2	Prostaglandin E2
POMC	Pro-opiomelanocortin
PPARγ	Peroxisome proliferator-activated receptor-γ
PRRs	Pattern recognition receptors
PVN	Paraventricular nucleus
PYD	Pyrin domain
RANTES	Regulated on activation, normal T cell expressed and secreted
RD	Regular diet

RD-NI	Non-injected WT mice fed a regular diet
ROS	Reactive oxigen species
rpm	Revolutions per minute
SN	Substantia nigra
SNpc	Substantia nigra pars compacta
SNpr	Substantia nigra pars reticulata
STN	Subthalamic nucleus
TAK 1	Transforming grow factor-β-activated protein kinase 1
TBK1	TANK-binding kinase 1
TGF-β	Transforming growth factor-β
TH	Tyrosine-hydroxylase
TIR	Toll-interleukin-1 receptor
TLR`s	Toll-like receptors
TNF-α	Tumor necrosis factor-α
TNFR1	TNF receptor 1
TNFR2	TNF receptor 2
TRAF6	Tumor necrosis factor receptor-associated factor 6
TSH	Thyroid stimulating hormone
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VLA-4	Very late antigen-4
VMAT-2	Vesicular monoamine transporter-2
VTA	Ventral tegmental area
WHO	World Health Organisation
WT	Wild type
α-MSH	α-Melanocyte-stimulating hormone
·OH	Hydroxyl radical

6-OHDA	6-hydroxydopamine

Contents

1. Introduction

The activation of innate and adaptive immune system has been associated with the progression of many neurodegenerative diseases [1, 2]. The activation of peripheral immune cells by intraperitoneal (i.p.) LPS injection induces the synthesis and release of proinflammatory cytokines in peripheral organs and within the brain [3]. The connection between the peripheral immune response and the neuroinflammatory process in the CNS will be discussed in the chapters 1.1-1.6.

The sustained neuroinflammatory process in the brain has been shown to be involved in the pathobiology of Parkinson`s disease [4]. The function of dopamine neurons and their susceptibility to neurodegeneration by the activation of microglia resident immune cells will be described in chapters 1.7-1.10.

Although obesity has been initially described as the excessive storage of fat, it has been also recognized as a pathological condition that contributes to many metabolic disorders and the production of proinflammatory cytokines [5]. Excess accumulation of fat causes a chronic state of low-grade chronic inflammation since it has been reported that food high in fat and sugar can act as an inflammatory component triggering the activation of the innate immune system [6]. Thus, it is proposed that there might be a connection among obesity-induced metabolic disorders, inflammation and neurodegenerative diseases [7-9]. High-fat diet-related weight gain contributes to leptin and insulin resistance that might alter the proper function of the dopamine system [10-13]. Effects of obesity-related metabolic disorders such as leptin and insulin resistance on dopaminergic function will be described in chapter 1.11.

1.1 The immune system and inflammation

1.1.1 The arms of the immune system

One of the aspects of maintaining physiological homeostasis and normal functioning of an organism is the presence of a defense system that is active against external pathogens. Such protection is enabled by an immune system comprised of cells and molecules that are actively involved in the recognition and neutralization of foreign agents, carried out in a highly coordinated process. Based on the level of the immune

system and specificity in recognizing hostile microorganisms, immune cells are divided into two major groups: innate and adaptive immune cells [14, 15].

The first reaction of the immune system towards the invading pathogens is mediated by the activation of the innate immune system. The innate immune system is phylogenetically older and this line of defense is generally defined as a nonspecific response. The innate immune system is only able to recognize common structural components of microorganisms and therefore fails to differentiate other key features that are specific for each pathogen alone. Part of the innate immune system includes existence of physical barriers (epithelial cells) that have the function of preventing entry of pathogens followed by the activation of various phagocytic cells [15].

The second line of the immune response is mediated by set of immune cells (T and B lymphocytes), which target pathogens in more specific way. Lymphocytes are specific in their ability to recognize a pathogen`s unique structural molecules and to memorize the previous pathological event in order to adapt their response for the next coming infection and execute its action more swiftly. In that sense this specific immune system is named as the adaptive immune system [14].

The innate and adaptive immune systems are inter-connected in their action to eliminate invading pathogens by orchestrating their response in two ways: 1) once activated, the innate immune system stimulates the activation of the adaptive immune system and also influences the level of its response; 2) the adaptive immune system recognizes and marks pathogens for destruction by employing the innate immune cells to eliminate microorganisms, closing in that manner the full cycle of the immune response [14, 16].

1.1.2 The inflammatory response

The inflammatory response represents an early non-specific response of the organism to tissue damage or pathogen invasion. It is mainly driven by the activation of circulating leukocytes, which are also a part of the main surveillance system responsible for protection against invading pathogens [15].

As described previously in chapter 1.1.1, the first line of defense is exerted by a physical barrier (epithelial cells of the skin). The barrier is quite effective in keeping the foreign microorganisms isolated from the internal environment. If this barrier

becomes damaged and penetrable to the invading pathogens, such an event will trigger the activation of another set of innate immune cells: phagocytic cells (monocytes/macrophages, neutrophils and natural killer cells) that non-specifically would recognize and destroy pathogens by secreting cytotoxic components, which is followed by their digestion (phagocytosis) [17].

Non-specific recognition of foreign substances (antigens) present in circulation corresponds to the outer part of the cell wall of microorganisms. More specifically, it has been recognized that solely the existence of fragments of the bacterial cell membrane within the host system was effective for the development of an inflammatory reaction. Indeed, it has been observed by Pfeiffer in the 19th century that insoluble part of bacterial cells was sufficient to trigger the activation of the innate inflammatory response and this substance was termed as "endotoxin" (endo=within, in Greek) [18]. Endotoxin refers to a component found on the outer part of the cell wall of Gram-negative bacteria. It has been shown that its structure contains lipid and polysaccharide and therefore it was termed as lipopolysaccharide (LPS) [19]. The notion that endotoxin has to be recognized in a host by a specific receptor led to the discovery of the lipopolysacharide receptor in C3H mice, derived by the mutation of a single locus and named as *Lps* [20]. In 1970, macrophages were recognized as the primary cells of the innate immune system responsible for the recognition of LPS [21]. Since then, LPS has been regarded as a poison and pyrogen. On the other hand, this endotoxin has been shown not to be detrimental in absence of its binding protein (low-density-lipoprotein), present in the blood [22].

Activated innate immune cells sensitize adaptive immune cells by activation of a complement system that leads to the release of cytokines and interleukins (IL). Cytokines represent a large family of signaling molecules that are secreted by the cells of the innate and adaptive immune systems exerting autocrine, paracrine and endocrine effects on distant organs [23]. Interleukins belong to the same group of cytokines, but were subdivided as it was thought that these signaling molecules are primarily produced by lymphocytes.

The role of lymphocytes is in elimination of specific antigens through the activation of T lymphocytes, which is accomplished by the production of antibodies (the soluble form of antigen receptors) secreted by B lymphocytes. T lymphocytes are

divided into two groups based on the surface molecules expressed on their membrane: CD4 and CD8 [24]. The CD4 group of T lymphocytes are recognized as a T helper cells and they are generally subdivided into Th1 and Th2 cells, even though some more subtypes have been discovered (Th17, Th9) [24]. Th1 cells are involved in stimulation and activation of other immune cells, mostly related with autoimmune diseases, whereas Th2 cells are involved in the activation of B lymphocytes and the production of anti-inflammatory interleukins. Production of Th2 cell types is important in the elimination of excessive proinflammatory action of Th1. Secreted by innate immune cells, cytokines promote clonal differentiation of previously activated lymphocytes into their cytotoxic form or stimulate the secretion of antibodies. At the same time, activated T lymphocytes are also a great source of cytokines, which have purpose of activating other immune cells. When inflammation subsides, some of these activated lymphocytes stay activated for a longer period of time to provide protection against the re-invasion of microorganisms.

Inflammation that occurs in peripheral organs is characterized by increase in temperature, swelling, redness and pain. A hallmark of acute immune reaction is increased permeability of the vascular system and infiltration of the cytokine-releasing phagocytic cells to the site of injury. In most cases, this event is self-limiting and activated phagocytic cells undergo the process of apoptosis if the primary stimulus persists any longer. On the other hand, if the antigen is still present, this can lead to sustained macrophage activation, which is described as a hallmark of chronic inflammation [25].

1.1.3 Peripheral inflammation

Microorganisms and other pathogens that reach circulation can be rapidly recognized by circulating monocytes and local macrophages such as Kupffer`s cells in the liver [26]. This recognition process is accompanied by the activation of Toll-like receptors (TLR`s) that reside on the external membrane of immune cells. TLR`s are known as pattern recognition receptors (PRRs) as they recognize specific patterns, also referred to as pathogen associated molecular patterns (PAMP`s) presented on the cell surface or inside it. There are many different types of TLR`s [27], divided by their function to recognize specific type of PAMP`s and the most important one that will be described further in the text is TLR4 [28]. TLR4, expressed by innate immune cells, mediates recognition of LPS [28]. Recognition of LPS is followed by

recruitment of the nuclear transcription factor (NF-κB) and activation of the IL-1 signaling pathway [29, 30]. As a result of TLR4 activation, certain proinflammatory cytokines such as IL-1, IL-6, tumor necrosis factor-α (TNF-α) are synthesized and released into circulation [28]. Cytokines are synthesized in a small amount (nano- to picomolar concentrations) and act in the autocrine manner, modulating the function of the same cell where they are produced, or in the paracrine manner, by interacting with adjacent cells [31]. They are also capable of inducing their own synthesis or release and the production of other cytokines (e.g. TNF-α and IL-6). At the same time, proinflammatory cytokines antagonize their inflammatory effect by inducing the synthesis of anti-inflammatory cytokines such as IL-10, interleukin-1 receptor antagonist (IL-1ra) [32].

Although cytokines are not long-range signaling factors and are not able to penetrate the blood-brain barrier (BBB) passively, they are still able to signal the brain through brain areas that are devoid of BBB and indirectly through the synthesis of prostaglandins. Generally it is accepted that cytokines may signal the brain in humoral and neural ways.

1.2 Communication between cytokines and the brain

There have been recognized four main routes of cytokine-to-brain communication: 1) cytokines synthesized in the periphery bind to the receptors of the *vagus* nerve [33], 2) secreted by the circulating immune cells, cytokines induce their own production and the induction of liposoluble molecules such as nitric oxide (NO) and prostaglandin E2 (PGE2) by endothelial cells and resident immune cells that are in contact with the BBB [34], 3) transmission into the brain from the area of circumventricular organs (CVO`s) [35] and 4) diffusion through the BBB by the mechanism of saturable transport [36].

1.2.1 Cytokine-induced neural communication through the *vagus* nerve

Neuronal communication between cytokines and the brain parenchyma is established through afferent projections of *vagus* nerves. The *vagus* nerve is connected to many different organs in the periphery, conveying the information from and back to central nervous system (CNS). At the same time, information about the condition of peripheral organs is sent back to CNS via the *vagus* nerve [37].

In rodent models of LPS-induced inflammation in peritoneal cavity, the ends of *vagus* nerves react by production of proinflammatory factors (e.g. IL-1β), since their perineural sheaths contain macrophages and dendric cells [38]. Neurons of *vagus* nerves also express receptors for IL-1β, so in that way signals from the periphery are transmitted to the brain [39]. The IL-1β-induced neuronal transmission is followed by the activation of several brain areas such as hypothalamus, brainstem and limbic system [39]. The evidence that the *vagus* nerve is involved in the transmission of peripheral inflammation to the brain emerged from animal models in which the *vagus* nerve was surgically ablated [40]. In that experiment, vagotomized animals did not experience sickness behavior (social withdrawal) with peripheral administration of LPS or IL-1β [40] but failed to show resistance upon intracerebroventricular (i.c.v.) IL-1β injection, leading to the conclusion that vagotomy itself does not alter the sensitivity of the brain to immune system challenges [41]. Even though vagotomized animals showed decreased signs of sickness behavior (similar to depressive-like behavior) after peripheral inflammation, it has been noticed that these animals were still able to develop fever through synthesis of prostaglandins in one part of the brain (organum vasculosum of the laminae terminalis) [42], which implies that there are other pathways besides neural ones that may propagate the immune reaction in the brain.

1.2.2 Humoral communication between cytokines and the brain

The humoral signaling pathway is another, yet slower way of transmission of peripheral inflammation to the CNS, characterized by the activation of the hypothalamic-pituitary-adrenal axis (HPA). It is considered that CNS recognizes peripheral inflammatory events by receiving information from secreted cytokines, through the activation of the HPA axis [43]. In connection with the previous statement it was previously demonstrated that hypophysectomized rats [44] and mice [45] failed to show IL-1 induced HPA activation [46]. Upon immune challenge in the periphery, cytokines are being synthesized by circulating immune cells. Released from the innate immune cells, cytokines such as IL-1, TNF-α and IL-6 stimulate the synthesis of PGE2 that is mediated by cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), which catalyzes production of NO [47]. As a result, PGE2 and NO are synthesized and released into the brain parenchyma. More specifically, PGE2 diffuses into the hypothalamus and binds to its receptors (EP3

and EP4 receptors) that are located in neurons of paraventricular nucleus (PVN) [48].

Subsequently, the corticotropin-releasing hormone (CRH) is synthesized [46] and transported to the pituitary gland where it stimulates the release of the adrenocorticotropic hormone (ACTH) in the blood. Recognized by its receptors on adrenal gland, ACTH further stimulates the secretion of glucocorticoids (e.g. cortisol). Circulating glucocorticoids act as a modulator of immune response by suppressing the further release of cytokines and by stimulating the anti-inflammatory action of Th2 cells [49]. Cytokines are also able to directly stimulate the HPA axis by binding to their receptors that are expressed in pituitary and adrenal gland, contributing to the elevated levels of glucocorticoids during chronic inflammation [50].

The neural and humoral pathways are two main routes of communication between peripheral inflammation and the brain. Even though these two pathways are connected in a manner that is still not fully understood, stimulation of both results in an expression of IL-1 in brain parenchyma. Produced by the resident macrophages in the brain, IL-1 is a proinflammatory cytokine that was shown to be implicated in many different neurodegenerative diseases [51].

1.3 Peripheral inflammation and activation of immune system in the BBB

The CNS is known as an organ that is largely protected by the BBB that provides isolation and protection from pathogens, proteins and immune cells [52]. The BBB is a physical barrier separating the brain from the systemic blood and it is quite complex in its structure. This structure includes several components: endothelial cells, basal lamina, astrocytes, pericytes (contractile cells) and perivascular macrophages [53]. Brain endothelial cells form tight junctions that regulate entry of endogenous compounds from the periphery. The tight junctions are enabled by the presence of integral membrane proteins such as claudin, occludin and junctional adhesion molecule-A (JAM-A) [54]. Endothelial cells are polarized cells and their cell membrane is quite unique in that one side is exposed to the blood and the inner side is connected to the brain parenchyma. This type of membrane expresses different kinds of transporters that allow communication between the brain and

circulating blood [55]. Endothelial cells are known to express the following types of TLR: TLR2, TLR4 [56], and TLR3, TLR6 [57].

Presence of the inflammatory factors in the peripheral circulation or produced by the brain endothelial cells may cause leakage of the BBB. Production of reactive oxigen species (ROS) from monocytes in *in vitro* studies, was suggested to contribute to increased BBB permeability [58]. Production of inflammatory cytokines (e.g. IL-1, IL-6 and TNF-α) by resident macrophages, endothelilal cells or transported from the blood are shown to increase permeability of BBB [59], but the exact mechanism is still unclear. More specifically, it has been shown that endothelial cells are more responsible as a source of circulating cytokines and sustainable inflammation in the brain, rather than microglial cells that reside next to the BBB [60]. Along with PGE2, IL-1β may be synthesized by local macrophages [61] and then promote its own production by pericytes in the region of CVO`s, choroid plexus [62], endothelial cells of CVO`s [63] and the BBB [64]. It has been shown that IL-1β expression in the CNS induces reversible breakdown of the BBB [65]. Endothelial cells express also several adhesion molecules such as lymphocyte functional antigen-1 (LFA-1), intercellular adhesion molecule-1 (ICAM-1), very late antigen-4 (VLA-4) and vascular cell adhesion molecule-1 (VCAM-1) that enable migration and adhesion of leukocytes to the site of injury [66]. Thus, stimulated by inflammatory factors (e.g. TNF-α or IL-1) or LPS, adhesion molecules are shown to enable lymphocyte trafficking across the BBB [67]. Interestingly it has been shown that JAM-A acts as an adhesion molecule for the infiltrating leukocytes in inflammatory conditions [68], contributing in that manner to higher permeability of the BBB. In *in vitro* studies conducted in primary cell culture of pericytes, TNF-α has been shown to induce the release of matrix metalloproteinase-9 (MMP-9) [69] a zinc-dependent endopeptidase, the increased level of which has been associated with BBB breakdown [70]. In post-mortem studies conducted on patients suffering from Parkinson`s disease, the BBB [71] has been observed to be disrupted with alteration of the blood vessels [72], caused by the high levels of circulating vascular enothelial growth factor (VEGF) [73]. VEGF is a very important growth factor involved in angiogenesis, but it also has been shown to cause BBB leakage upon intravenous administration in rodents [74].

Overall, mounting evidence suggests that peripheral inflammatory cytokines interact with brain endothelial cells of CVO`s and choroid plexus. These afterwards induce the production of proinflammatory cytokines (e.g. IL-1) in brain parenchyma. These chains of events represent a general pathway of communication between the periphery and the brain in the inflammatory process (figure 1.1).

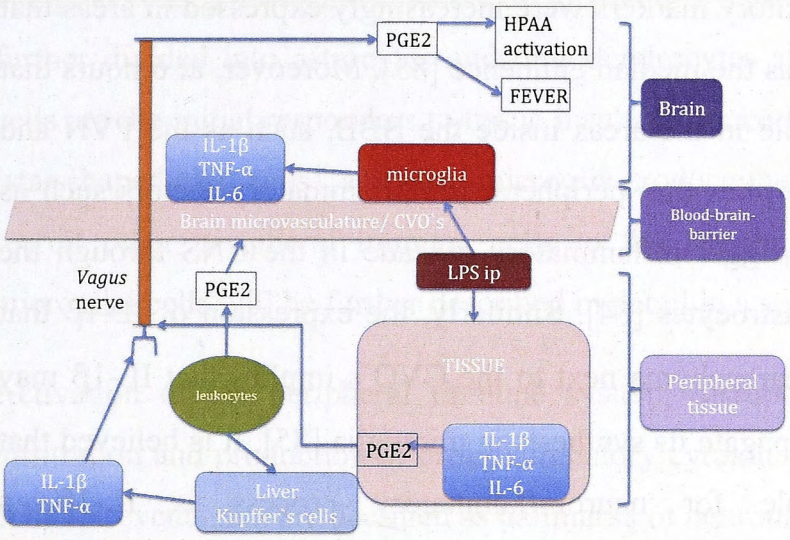


Figure 1.1: Schematic representation of signaling pathways between peripheral organs and the brain.

1.4 Diffusion of peripheral cytokines and lymphocytes into brain parenchyma and activation of resident immune cells

Even though the CNS has been considered as an "immune privileged" organ in the past because of the presence of the BBB [75], upon immune challenge, cells such as leukocytes may still penetrate the BBB and secreted cytokines may enter the brain parenchyma. Since the first two routes of cytokines trafficking have already been described, the main focus of this section will be to describe the process of cytokines transmission into the brain via CVO`s and the mechanism of saturable transport.

Proinflammatory cytokines produced in the periphery such as TNF-α, IL-1β and IL-6 [76] can cross the BBB via specific saturable transporters in endothelial cells [77]. Cytokines are also able to diffuse into the brain in the areas that are less protected by the BBB (CVO`s and choroid plexus) [78], as it has been shown that cytokines such as TNF-α and IL-1β were expressed within CVO`s [79, 80]. Diffused cytokines (e.g. IL-1β) propagate their own production by activation of TLR on innate immune cells in the CVO`s [81]. TNF-α is then able to activate NF-kB of nearby microglial cells to synthesize inflammatory factors that can further spread across brain parenchyma.

In a rodent model of peripheral LPS-induced inflammation, it has been shown that few factors are elevated within brain parenchyma: COX-2, iNOS and IL-1β mRNA [82]. More specifically, the expression of IL-1β mRNA and iNOS mRNA was initially induced in the areas that are outside the BBB such as choroid plexus, meninges and vasculature after 2 hours of LPS injection [83]. Later at 6 hours from LPS injection, these inflammatory markers were increasingly expressed in areas that are outside of the BBB such as the median eminence [83]. Moreover, at 6 hours that expression was also noticeable in the areas inside the BBB, such as the PVN and arcuate nucleus [83]. This implies that peripheral proinflammatory factors such as NO and prostaglandins can trigger inflammatory cascade in the CNS through the activation of microglia and astrocytes [84]. Similarly, the expression of IL-1β that has been observed in brain parenchyma next to the CVO`s implies that IL-1β may diffuse into the brain and propagate its synthesis in microglia [85]. It is believed that a key element responsible for neuroinflammatory process is IL-1β, a proinflammatory cytokine that is upregulated in most neurodegenerative disorders (e.g. Parkinson`s disease, Alzheimer disease) [86-88].

Trafficking of immune cells through BBB is achieved by process of diapedesis. The passage that leukocytes use to penetrate into the brain is in the place of the postcapillary venules [89]. This process includes many steps that involve adhesion to the endothelial cells of BBB with their transportation across the wall of blood vessels. Infiltration of leukocytes includes activation of many adhesion molecules and signaling pathways. Leukocyte trafficking into the BBB is achieved in a two-step process. The first involves expression of selectins on blood vessels that attract carbohydrate ligand on the leukocytes cell membrane. As a result of this cellular binding, leukocytes express integrins on their cellular membrane [90], which strengthens adhesion between the vascular surface and the leukocytes. At the very end of this two-step process, leukocytes are recruited through the BBB. In contrast to the peripheral inflammation, during brain inflammation there is only a small fraction of peripheral neutrophiles crossing the BBB [91].

Along with peripheral organs being under protection by immune cells, the brain parenchyma is also under constant surveillance by resident immune cells situated at the walls of cerebral blood vesels and inside the parenchyma [92]. Activation of

resident immune cells in the brain as a response to threat is a process defined as a neuroinflammation. Macrophages and dendritic cells residing close to the blood vessels of meninges and choroid plexus, represent the first line of defense against invading pathogens. If the inflammatory insult eventually crosses the BBB then another set of cells will be activated, such as brain endothelial cells, macrophages and glial cells. These glial cells are divided into two groups: 1) macroglia, which are further divided into astrocytes and oligodendrocytes and 2) microglial cells. Glial cells are the initial responders to tissue insult. In concert with surrounding astrocytes (star-shaped glial cells), activated microglia produce the majority of chemokines that recruit other peripheral immune cells to the site of injury [93]. The action of microglial cells will be further described in detail in a separate chapter.

Activation of the peripheral immune system, disruption of the BBB, leukocyte infiltration and production of proinflammatory cytokines by brain resident immnune cells are events that are decribed as hallmarks of neuronal degeneration [94].

1.5 Interaction between LPS-induced cytokines and BBB

One of the most common stimuli that has been used for the investigation of the CNS response to inflammation is LPS [95], usually injected intravenously or intraperitoneally. The purpose of using systemic LPS injection is to mimic sepsis and infectious challenges.

While peripheral inflammation usually starts in a specific organ, LPS-induced systemic inflammation rapidly activates TLR that are present on the vasculature and parenchyma of different organs [96]. It was shown that the half-life of intravenous injected LPS in blood is approximately 30 minutes and most of it is reabsorbed by abdominal organs (spleen and liver) and lungs [97]. More specifically, LPS has been found in lysosomes of Kupffer`s cells in the liver, and in macrophages and leukocytes of the spleen [97]. Remaining residue of LPS is cleared out from the system with a half-life of 12 hours [97]. In case of intraperitoneal injection, LPS reaches the blood within 15 minutes [98], but is not able to breach the BBB. Instead it was found to bind to endothelial cells of the brain [56]. Administrated directly into the primary culture of brain microvascular endothelial cells, LPS has been shown to dysregulate tight junctions between the cells suggesting that LPS might increase BBB permeability, allowing the entrance of external insults [99].

Even though circulating LPS has a limited access to the BBB, it can still bind to TLR`s present on microglia and macrophages from CVO`s, choroid plexus and leptomeninges of the brain [100]. For instance, it has been shown that intraperitoneal injection of LPS (1 mg/kg) can cause activation of microglial cells in the brain with concomitant production of proinflammatory cytokines [101]. Activation of microglia through TLR`s depends on routes of LPS administration. If LPS is administrated directly into the brain, then microglia will be activated through TLR4-MyD88 pathway [102]. On the other hand, activation of microglia by systemic LPS depends on the proinflammatory cytokines such as IL-1β [96, 103] and TNF-α [81]. Although it has been reported that TLR4 is expressed by astrocytes [104], endothelial cell [105] and neurons [106], the CNS immune response is mainly mediated by microglia [102].

Systemic LPS can also mediate its action on CNS by stimulation of the afferent projection of the *vagus* nerve [38]. Macrophages and dendritic cells that reside in perineural sheaths of *vagus* nerve produce IL-1 when stimulated by LPS [38]. As a result, IL-1 binds to its receptors located on the *vagus* nerve [39], resulting in activation of *vagal* afferent projections. In this manner, an inflammatory response from the periphery is delivered to the brain in very short period of time.

Interaction between systemic LPS and CNS may also be established indirectly, through the cytokines that are synthesized in the periphery by local lymphocytes. Released from the peripheral immune cells upon LPS stimulation, IL-1β is further synthesized from macrophage-like cells, located in CVO`s and choroid plexus [85]. Produced in that way, IL-1β may interact with its receptors and propagate its further action in the brain.

During acute neuroinflammation, damaged neurons tend to recover over a short period of time, but the process of chronic neuroinflammation tends to persist long after an initial insult has begun [107]. For instance, in acute model performed on monkeys injected intramuscularly with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), loss of dopamine neurons were recorded in substantia nigra, striatum and pallidum [108]. Despite having substantial loss of dopamine neurons, these animals recovered their locomotor activity due to increased number of dopaminergic and serotonergic fibers that compensated for the loss of neurons [108].

In another study, single systemic LPS injection induced delayed and progressive loss of dopamine neurons in male mice that was accompanied by elevated level of proinflammatory cytokines, seven months after the initial insult [109]. Sustained inflammation leads to activation of peripheral or CNS resident immune cells and it is considered that this long-term self-perpetuating process has a detrimental effect on neuronal survival in different neurodegenerative diseases.

1.6 Inflammatory signaling mediators in CNS

During peripheral inflammation there are certain mediators that are expressed in peripheral organs and glial cells of the brain. Early response to immune activation is characterized at first by secretion of inflammatory markers such as cytokines (e.g. IL-1, IL-6, IL-18 and TNF-α) and chemokines (cytokines that enable chemoattraction between cells), followed by others, such as NO, eicosanoids and ROS. The interleukin-1 group of cytokines belongs to a large family of proinflammatory cytokines, produced during infection/inflammation by local lymphocytes [110]. Most notable cytokines from this family that will be discussed further in the text are: IL-β, IL-1α, IL-1ra, IL-18 and some others interleukins that do not belong to this group, such as IL-6. Caspase-1 is the enzyme that is involved in cleavage of inactive IL-1β, IL-18 and IL-33 into their mature bioactive form [111]. Below, there is a brief description of cytokines that are relevant factors to the IL-1 pathway and some others, such as TNF-α and IL-6.

1.6.1 Caspase-1 (Casp-1)

Caspase-1 is an enzyme that cleaves proinflammatory cytokines IL-1β (pro-IL-1β), IL-18 and possible IL-33 into their active form [111]. There are two types of caspase sub-families: inflammatory and apoptotic. It is considered that caspase-1 is part of inflammatory group. Besides having part in the activation of cytokines, caspase-1 is also a part of the programmed cell death signaling pathway named as pyroptosis, an intracellular process of mediated cell death of infected macrophages that contributes to the release of IL-1β [112].

Caspase-1 is synthesized as an inactive precursor (pro-caspase-1) and it is cleaved in its active form by a mulitiprotein complex (inflammasome) [113]. Activation of the inflammasome is followed by the stimulation of TLR`s or purinergic receptors (P2X7) expressed on the cell membrane of cyrculating monocytes and macrophages.

Activation of purinergic receptors by adenosine triphosphate (ATP) or LPS causes caspase-1 dependent cell death of macrophages with concominant release of proinflammatory cytokines [114]. More specifically, ATP exposure causes only modest release of IL-1β, whereas LPS is necessary for maturation of this cytokine and cell death [115].

Inhibition of caspase-1 in inflammatory processes has a positive effect on neuronal survival. It was demonstrated that administration of caspase-1 inhibitor reduces brain injury followed by ischemia [116]. More specifically, caspase-1 knockout mice showed significant resistance to MPTP-induced loss of dopamine neurons [117]. The MPTP is neurotoxin that is often used in animal model of Parkinon`s disease due to its capability to selectively destroy dopaminergic neurons [117]. Therefore, caspase-1 is considered as a valuable therapeutic target in the treatment of brain diseases since many neurodegenerative disorders are characterised by a high-level of neuroinflammation [118].

1.6.2 Interleukin-1β (IL-1β)

IL-1β is a proinflammatory cytokine, released from immune cells in response to acute and chronic phases of systemic injury or disease. Except for the caspase-1 dependent process of IL-1β activation, which represents the essential pathway of IL-1β maturation, this inteleukin can be also processed extracellularly into its active form by proteinase-3, elastase, matrix metalloprotease-9 (degrades extracellular macromolecules) and granzyme A [119-122]. IL-1 exerts pleiotropic effects on surrounding cells of peripheral organs and the brain.

IL-1β is known to have several physiological functions within the CNS. Levels of IL-1β during prenatal and postnatal periods of the brain development were shown to be elevated in the cortical part of the brain [123], while in adulthood IL-1β is sustained at a very low level [124]. Relevance of IL-1 in brain development has been shown on a example of the development of the dopaminergic system where IL-1 played a key role in proliferation and concomitant differentiation of mesencephalic dopaminergic progenitors [125]. In normal conditions low quantities of IL-1 are synthesized and its function in the CNS is to maintain long-term potentiation, sleep and memory [126]. IL-1 has a role in modulating cell membrane potential. It can increase neuronal activity through enhancement of N-methyl-D-aspartate (NMDA)

current [127]. On the other hand, it can trigger hyperpolarization by enhancing inhibition of gamma-aminobutyric acid (GABA) [128]. Therefore, physiological levels of IL-1 (pM range) are necessary for maintaining long-term potentiation on neuronal membranes [129]. Secreted in large quantities, IL-1 exerts endocrine function by increasing the secretion of corticotropin releasing factor (CRF), ACTH, cortisol and thyroid stimulating hormone (TSH) from the pituitary [130-132].

Interleukin-1 is a key mediator of inflammatory processes in the peripheral organs. In the periphery, the production of IL-1β is regulated by monocytes, macrophages, B lymphocytes and natural killer cells. Secreted in a small amount, IL-1 functions as a mediator of local inflammation by stimulating endothelial cells to secret integrins that contribute to adhesion of circulating leukocytes [133]. Produced by peripheral limphocytes, IL-1β stimulates the synthesis of PGE2 in blood vessels that can penetrate in the brain and activate neurons in substantia nigra [134]. IL-1β that reaches the brain, produced from peripheral neutrophiles and monocytes, activates microgial cells to further increase its synthesis and release [135].

In the brain, action of IL-1β is mediated through activation of glial cells (microglia, astrocytes and oligodendrocytes), where it contributes to their proliferation and production of inflammatory factors [136]. Produced and secreted in the brain, IL-1β stimulates infiltration of peripheral immune cells into the brain by repressing the brain resistance to recruitment of leukocytes [137, 138]. Upon binding to its receptor, IL-1 receptor 1 (IL-1R1), IL-1 orchestrates the synthesis and induces release of proinflammatory factors from glial cells and activates the NF-kB signaling pathway [139]. IL-1 also has a function of modifying the physiological function of local neurons through the activation of the sphingomyelinase (nSMase) signaling pathway [140, 141]. It has been reported that intranigral injection of a recombinant adenovirus that expressed IL-1β caused progressive loss of dopamine neurons in substantia nigra pars compacta (SNpc), just three weeks after the exposure accompanied with motor disabilities and activation of microglia [142]. In a state of CNS inflammation, inhibition of IL-1 has been shown to ameliorate the inflammatory process and improve neuronal survival [143].

All these reported data suggest that infiltration of inflammatory markers into the brain parenchyma, especially IL-1β as a result of peripheral inflammation, may

increase inflammation in substantia nigra and consequently have a deleterious effect on functionality of dopamine neurons.

1.6.3 Interleukin-1α (IL-1α)

Interleukin-1α has a function as an autocrine growth factor [144]. Even though the exact mechanism of its activation remains unknown, this cytokine may be activated by calpain (Ca^{2+} activated cystein-protease) that is associated with cell membranes [145]. Nevertheless, its precursor form is biologically active and contributes to the cellular differentiation of endothelial and ectodermal cells [146]. IL-1α and its precursor are expressed in the epithelial cell under normal conditions and their function is impaired by the intracellular form of interleukin-1 receptor antagonist (icIL-1ra). Except for its intracellular form, IL-1α is also found on the cell membrane of monocytes and B lymhocytes [147] where it plays a major role in the inflammatory process. IL-1α was expressed by astrocytes in a variety of brain areas (striatum, substantia nigra and ventral tegmental area) during peripheral MPTP-induced inflammation, and it was proposed to contribute to recovery of dopaminergic neurons by inducing axonal growth [148]. Peripheral injection of IL-1α was implicated in activation of the HPA axis and development of fever [149].

1.6.4 Interleukin-1 receptor antagonist (IL-1ra)

IL-1ra is a cytokine that belongs to interleukin-1 family. It has been recognized for its function of inhibiting IL-1β [150] action by binding to its receptor (IL-1R1) on the membrane cell surface, without eliciting second messenger signaling [151]. IL-1ra is secreted by a variety of cell types such as immune cells [152], endothelial cells [153], adipocytes [154].

Since IL-1ra functions as an inhibitor, its production is correlated with the synthesis of IL-1 [155]. IL-1β and IL-1α signaling pathways are conducted through IL-1R1 that resides on the cell membrane, and IL-1R1 associates with IL-1 receptor accessory protein (IL-1RAcP). Under normal conditions the ratio between IL-1ra and IL-1 is close to 1 [156]. In a state of inflammation it has been estimated that to effectively block the IL-1-induced activation, it is necessary that the ratio between IL-1ra/IL-1 is approximately 25:1 [157]. It has been shown that systemic administration of IL-1ra protected dopamine neurons in SNpc from exposure to polyinosinic:polycytidylic acid induced inflammation [158]. Also in *in vivo* studies

with rats, subcutaneous administration of IL-1ra attenuated dopamine cell loss in experiment with single intranigral injection of LPS into substantia nigra [159]. Upon brain injury, IL-1, IL-1R1 and caspase-1 are actively synthesized and produced by microglia [160], but during severe neuroinflammation an efficient blockade exerted by IL-1ra might not occur.

1.6.5 Tumor necrosis factor (TNF)

TNF is one of the main mediators of acute phase of inflammatory response to bacterial infection. It is among the first cytokines detected in the circulation after systemic inflammation with LPS [161]. Except being mostly related with the activation of the inflammatory signaling pathway, it is also involved in the apoptotic process, cellular proliferation and differentiation [162]. There are many TNF-related cytokines, but the most known are TNF-α and TNF-β. TNF is synthesized as a transmembrane homotrimer and it may apear in soluble form once cleaved by TNF-α converting enzyme. These two active forms exert their action on other surounding cells via their receptors: TNF receptor 1 (TNFR1) and TNF receptor 2 (TNFR2) [163]. Soluble form of TNF preferably binds to TNFR1 and transduces proinflammatory stimuli [164]. Activation of TNFR2 is accomplished via the transmembrane form of TNF and it is considered to promote proinflammatory and pro-survival signaling pathways [165].

In connection with IL-1, TNF stimulates the endothelial cell of the BBB to express membrane adhesion molecules and chemokines. During an *in vivo* inflammatory process, it has been shown that in relation with IL-1β, TNF is capable of increasing BBB permeability by activation of local endothelial cells and astrocytes to produce chemokines, which enables localy circulating leukocytes to infiltrate into CNS parenchyma [166]. In endothelial cells, TNF induces activation of COX-2 that in turn synthesize PGE2 and also induces iNOS which increases the NO. Within the brain, TNF meadiates activation of astrocytes and microglia to promote their differentiation [167]. Acting through TNFR1, cyrculating TNF mediates activation of the NF-κB inflammatory signaling pathway and ceramide/sphingomyelinase pathways, which leads to neuronal degeneration [168, 169]. Elevated plasma levels of TNF-α and its receptor are reported in Parkinson`s diseased patients and were suggested to contribute to the pathogenesis of this disease [170, 171].

1.6.6 Interleukin 6 (IL-6)

The interleukin-6 is a cytokine secreted by macrophages and T cells [172, 173]. This cytokine acts both as proinflammatory and anti-inflammatory interleukin by exerting its function through two receptors: soluble form IL-6R and IL-6R-α. Upon binding to the receptors, signal transduction is followed by activation of the JAK-STAT3 signaling pathway with concomitant expression of genes such as monocyte chemoattractant protein-1 (MCP-1) [174]. It also acts on the HPA axis and stimulates secretion of catecholamines with dysregulation of glucocorticoids [175]. Under normal conditions in the brain, this interleukin plays neurothophic and neuroprotective roles [176], acting as an inducer of brain-derived neurotrophic factor (BDNF) [177].

Following inflammatory challenge (e.g. LPS) and brain injury, the expression of IL-6 is highly up-regulated [175, 178]. In the brain, IL-6 causes activation of microglia and astrocytes [179]. Its production is mediated through several signaling pathways including one regulated by TNF-α [180]. More specifically, delayed activation of IL-6 has been shown to be dependent on TNF-α activation via TNFR1 [181].

Anti-inflammatory activity of IL-6 comes from its ability to control and decrease cytokines production in activated immune cells [182]. More specifically, IL-6 has been shown to deactivate the production of TNF-α and to induce of secretion of IL-1ra [179]. It has been demonstrated that increased levels of TNF-α and IL-6 showed protective effect against methamphetamine- induced cell death of microglia through activation of the JAK-STAT3 signaling pathway [183]. Similarly in *in vitro* studies, IL-6 has shown a protective role in the survival of midbrain dopaminergic neurons from 1-methyl-4-phenylpyridinium (MPP (+)) induced neurodegeneration [184].

1.6.7 Interleukin 18 (IL-18)

IL-18 is another proinflammatory cytokine released from immune cells after the recognition of certain pathogens. It has been first recognized as an inducer of interferon-γ (IFN-γ) [185]. IL-18 has been indentified as an important link between the innate and adaptive immune systems [186], as a potent stimulator in production of various chemokines and iNOS synthesis. Synthesized as an inactive precursor protein by varios cell types, IL-18 is cleaved in its active form by activation of caspase-1. Its precursor can be also be processed extracellularly by various enzymes,

such as serine protease, cathepsin G and elastase [187-189]. IL-18 exerts its action on adjacent cells via its receptor IL-18R that belongs to a family of TLR. Soluble protein IL-18 (IL-18BP) has been identified as a negative regulator of IL-18 action. Upon binding to its receptor, IL-18 mediates its action via recruitmet of adaptor myeloid differentiation factor (MyD88) with concominant activation of NF-κB [111]. In comparison to IL-1 and TNF-α, which are capable inducers of fever, IL-18 has no role in activation of COX-2 and therefore there is no production of PGE2 [190].

During the inflammatory process in the CNS, IL-18 is involved in microglia activation and infiltration of immune cells into the brain [191, 192]. In the brain, IL-18 is produced by microglia [193], ependymal cells and neurons of medial habenular nucleus [194], hippocampus, hypotalamus, cerebellum, cortex and striatum [195]. IL-18 is also produced by the adrenal cortical cells and pituitary gland [196], which suggest that this interleukin mediates the comunication between the endocrine and nervous system. Microglia are actively synthesizing IL-18 during inflammation and are also able to respond to this interleukin via IL-18R [196]. It has been shown that IL-18 deficient mice had impaired microglial activation [197] and therefore increased survival of dopamine neurons [191].

1.6.8 Chemokines

Chemokines represent a group of small cytokines. Their main function is regulation of leukocyte trafficking to the inflammatory site. According to their molecular stucture that contains cysteine residue, this large protein family was divided into subgroups (C (chemokines containing only one cysteine residue), CC (cysteine residues are connected together with a disulfide bond), CXC (disulfide bonds between cystein residues are separated with one amino acid (X)) and CX3C). They exert their function by binding to G protein-coupled receptors. There are only two chemokines expressed in the brain by neurons and atrocytes under normal conditions: fractalkine (CX3CL1) and SDF-1α (CXCL12) [198-200]. In a state of inflammation that occurs in the brain, chemokines are produced by resident immune cells such as microglia, astrocytes and endothelial cells of the BBB. Activated microglia by LPS secrete the following chemokines: chemokine IL-8 (CXCL8), interferon gamma-induced protein-10 (IP-10), macrophage inflammatory protein-1α (MIP-1α), MIP-1β, MCP-1 and regulated on activation, normal T cell expressed and

secreted (RANTES) [201]. In comparison with microglia, astrocytes exposed to LPS and other inflammatory insults produce the same group of chemokines except from MIP-1 and MIP-2 [202]. Chemokines expressed on the cell membrane of activated microglia stimulate their migration to the site of injury [203].

1.6.9 Eicosanoids

Eicosanoids are derivates of arachidonic acid and are found to play a major role in the pathogenesis of the CNS. Most important ones are the prostaglandins (PGD2, PGE2, PGF1a) converted from arachidonic acid by activation of COX-2. Prostaglandins are secreted at the site of injury, and it is reported that they might be synthesized by endothelial cells (PGE2) of CNS or transported though BBB from peripheral circulation [204]. It has been demonstrated that COX-2 and oxidative stress had a detrimental effect on survival of dopaminergic neurons, which was shown to be associated with high levels of PGE2 [205]. The main route of PGE2 to cause apoptosis of dopamine neurons has been shown to be via its receptor-endogenous pyrogen 1 (EP1) [206].

1.7 Dopaminergic neurons and their role in movement control

The predominant function of dopaminergic system is in control the smooth execution of limb movements and body posture. Other functions of the dopamine system include the control over the various behaviors, such as the procedural learning, emotions and cognition. This is enabled by the neurotransmitter dopamine that is synthesized and released in dopamine neurons of substantia nigra [207, 208].

Dopaminergic neurons are the main source of dopamine in the midbrain. Dopamine is catecholamine neurotransmitter and its synthesis is regulated by rate-limiting enzyme tyrosine-hydroxylase (TH) [209]. Dopaminergic neurons correspond to approximately 1% of total neurons. These neurons represent a heterogeneous group of cells, localized in three anatomical compartments: diencephalon, mesencephalon and olfactory bulb [210]. Approximately 90% reside in the ventral part of the mesencephalon. The mesencephalic dopaminergic system has been subdivided into three nominal systems as shown below (figure 1.2).

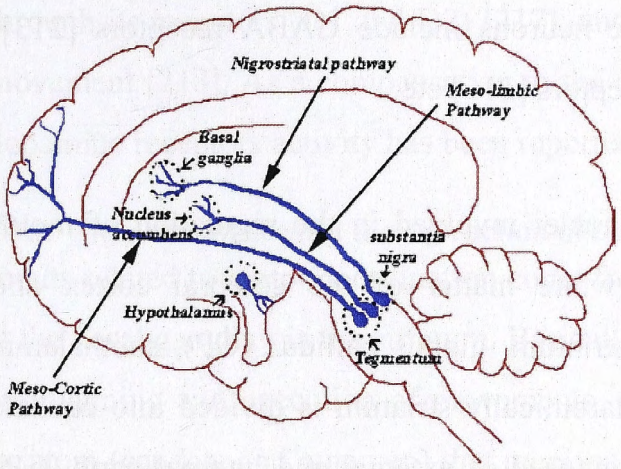


Figure 1.2: Schematic representation of the mesencephalic dopaminergic system [207].

1) The nigrostriatal system originates in SNpc and extends its fibers into the caudate-putamen region (also known as the dorsal striatum). This pathway plays a key role in control of voluntary motor movement.

2) The mesolimbic and 3) the mesocortical dopaminergic systems, arise from dopaminergic cells present in the ventral tegmental area (VTA). These two dopaminergic systems are involved in emotions such as motivation and reward. The neurons of the mesolimbic dopaminergic system originate in the VTA and project into the nucleus accumbens, the olfactory tubercle, the septum, amygdala and hippocampus. The dopamine neurons in the medial VTA, which have projections to the prefrontal, cingulated and perirhinal cortex constitute the mesocortical dopaminergic system. Because there is certain overlap between mesocortical and mesolimbic dopaminergic neurons, the two systems are often referred as the mesocorticolimbic system [211].

The physiological actions of dopamine neurons are established via membrane dopaminergic receptors. Dopamine receptors belong to the group of transmembrane G protein-coupled receptors and are divided according to their structure and function, into five subtypes: D1-like receptor subtype (D1 and D5) and D2- like receptors (D2, D3 and D4). D1- like receptors contain a Gs subunit that induces activation of adenylyl cyclase and therefore contributes to the increase of cAMP synthesis. On the other hand, D2- like receptors inhibit adenylyl cyclase and activate K^+ channels since their activity is regulated by a Gi subunit [212].

Other receptors expressed on dopamine neurons include GABA receptors [213], NMDA receptors [214], serotonergic receptors [215] etc.

1.7.1 Function of basal ganglia

The basal ganglia represents group of nuclei involved in the regulation of motor function [216]. It is situated in the white matter of the cerebral cortex and compromised of the following nuclei: striatum, globus pallidus (GP), subthalamic nucleus (STN) and substantia nigra. Anatomically striatum is divided into caudate and putamen. The globus pallidus is composed of external and internal parts (GPe and GPi). The substantia nigra contains dopaminergic neurons, and it is divided into SNpc and substantia nigra pars reticulata (SNpr). The main connections between described anatomical parts of the basal ganglia are schematically represented in figure 1.3.

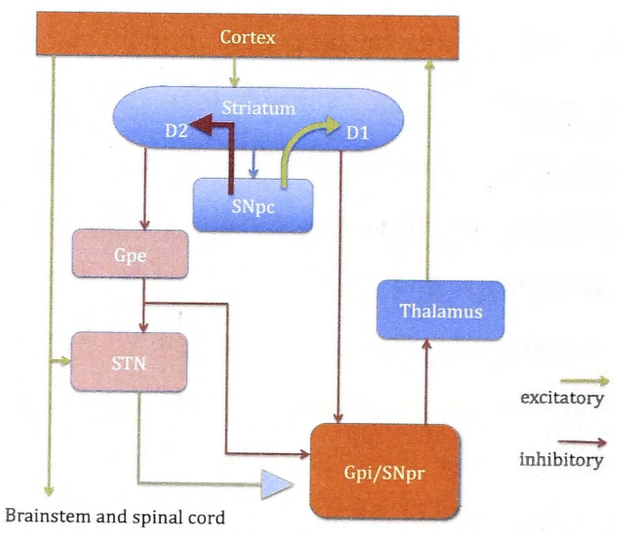


Figure 1.3: Schematic representation of circuits in the basal ganglia.

The basal ganglia is a part of a loop that transmits information from the cerebral cortex and back, via several major relay points: the striatum, the internal part of globus pallidus, the SNpr and the thalamus. In this process, the striatum receives information from the cortex, whereas GPi and SNpr send information back to the cortex. Input from the cortex is excitatory and it is established via glutaminergic neurons, while output is inhibitory and transmitted via GABA neurons. On the other hand, dopamine is involved in the regulation of neuronal firing in the striatum,

through its receptors (D1 and D2) [217], and in that way contributes to execution of movement [218]. As a compensation to the dysfunction of basal ganglia, increase in dopamine receptors activity has been reported [219].

1.7.2 Movement control through action of basal ganglia

Inputs related to motor coordination come from the motor cortical area and converge at the basal ganglia via the striatum. Basically, the cortical area, which is involved in the planning and execution of movements, projects to the striatum. Further on, the striatum (caudate and putamen) that receives this input signals to the globus pallidus. Information collected in the basal ganglia is transferred to the globus pallidus and then to the ipsilateral motor thalamus. From the thalamus, output is sent to the motor cortex [220].

Information that reaches the globus pallidus from the striatum are divided into two pathways: direct and indirect [221]. These two pathways exert opposite effects on motor activity and their dysregulation may explain problems seen in dysfunction of the basal ganglia.

In the direct pathway, information is gathered in the interior of the globus pallidus and then transduced to the thalamus and the motor cortex (figure 1.4). Signals from the motor cortex to the globus pallidus are sent via the glutamate neurotransmitter, which has the role of exerting an excitatory effect on striatal neurons. Neurons in the striatum then release a GABA that inhibits the cells of the globus pallidus and SNpc acting through the D1 receptor. Inhibition of GP cells leads to less inhibition of thalamic nuclei, which results in increased firing of neurons in the motor cortex. Eventually this will increase firing in the corticospinal tract and muscle movement [220].

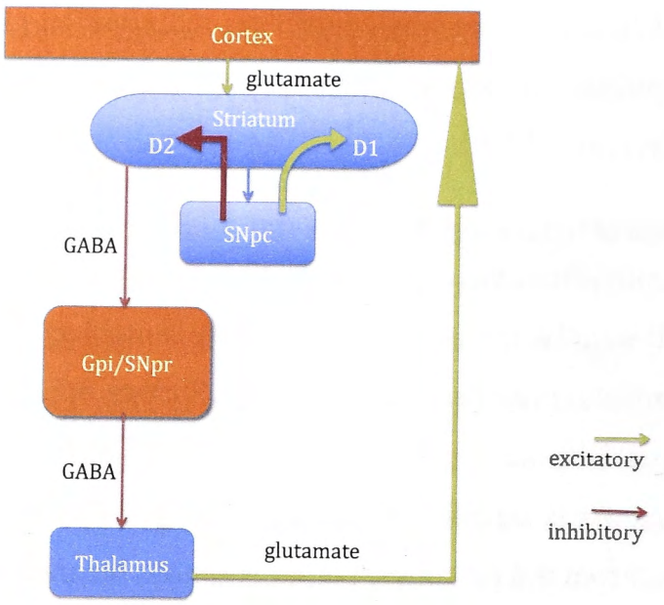


Figure 1.4: Representation of the direct pathway of the basal ganglia.

The indirect pathway is followed by neural signal transmission from the cortex to external part of the globus pallidus (figure 1.5). Before reaching the thalamus, signals from the external part of the globus pallidus are projected to subthalamic nuclei and SNpc, then backwards to the internal part of the globus pallidus. Signal transduction on these neurons is accomplished via D2 receptors. Signals from the internal part of globus pallidus are transmited to the thalamus and motor cortex. Signaling pathways in subthalamic nuclei, cortex and thalamus are mediated through glutamate, whereas in the globus pallidus and striatum are operated by GABA. Thus, neuronal communication can be stimulated or inhibited depending on the neurotransmitter that is involved. In the case of the indirect pathway, signal transduction via subthalamic nuclei results in a decrease of excitatory drive in the cortex [220].

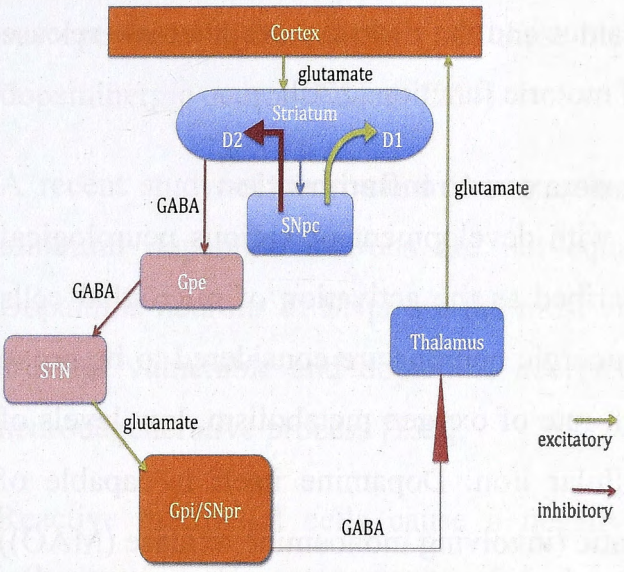


Figure 1.5: Representation of the indirect pathway of the basal ganglia.

Dopamine produced in SNpc is released in striatal neurons via nigrostriatal pathway, where it exerts its function by binding to D1 and D2 receptors. In the direct pathway, dopamine binds to D1 receptors and in that way exerts an excitatory effect on striatal cells. In concert with D2 receptors that are associated with the indirect pathway, dopamine has inhibitory effects on signaling in the striatum. According to this, released dopamine promotes motor activity via activation of the direct pathway and at the same time inhibits the indirect pathway that is responsible for turning down motor activity [220].

Loss of dopaminergic neurons in SNpc leads to decreased activity in the direct pathway with concomitant increase in activity of indirect pathways, which results in inhibition of thalamic-cortical output. Decreased activity in the direct pathway comes from loss of dopamine input from SNpc to striatum, which leads to decreased excitation of striatal neurons via D1 receptors. At the same time this decreased excitatory effect on striatum causes inhibition of GABA output to the internal globus pallidus and SNpr. Chronologically, loss of dopamine leads to inhibition of D2 in the striatum, which causes increase of GABA activity in the external part of the globus pallidus in the indirect pathway. This increased GABA activity in the external globus pallidus leads to less GABA release to the subthalamic nucleus, resulting in the increase of glutamate in SNpr. In all, reduction of GABA activity in the direct pathway with the increase of glutamate in the indirect pathway results in overall

increased induction of GABA in the thalamus and the decrease of glutamate release from the motor cortex leading to a loss of motoric function [220].

1.8 Susceptibility of dopaminergic neurons to inflammation

Neuroinflammation has been associated with development of various neurological disorders (e.g. Parkinson`s disease), described as the activation of microglial cells and depletion of certain neurons. Dopaminergic neurons are considered to be prone to oxidative stress [222] due to their high rate of oxygen metabolism, low levels of antioxidants and high levels of intracellular iron. Dopamine itself is capable of generating toxic ROS via both its enzymatic (involving monoamine oxidase (MAO)) and non-enzymatic catabolism involving free-transition metal ions like iron [223]. The oxidation of dopamine via MAO generates a wide range of toxic free radical species such as H_2O_2, semiquinones and quinones [224]. Free radical species have been shown to cause mitochondrial dysfunction by inhibiting complex I [225].

Presence of neuromelanin exerts a protective effect against oxidative damage of dopamine neurons [226]. Neuromelanin represents the accumulation of oxidative lipid derivates and altered proteins compiled in autophagic vacuoles [227]. In such a state, neuromelanin is further degraded by cellular lysosomes. In contrast, during the aging process these neuromelanin depositions may grow larger, as a consequence of limited clearance, and by interacting with other cellular components it may accumulate some of the toxic iron species which could exacerbate neuronal damage once neuromelanin is released from the cell [228].

Proinflammatory cytokines interfere with the synthesis of dopamine, its secretion and final reuptake into dopamine neurons [229]. Cytokines such as IL-6 and interferon-α (IFN-α) were shown to cause depletion of tetrahydrobiopterin (BH4) in CNS [230, 231], an essential enzyme co-factor that is important for the proper function of the tyrosine-hydroxylase enzyme. Dopamine reuptake into the vesicles of pre-synaptic dopamine neurons is dependent upon vesicular monoamine transporter-2 (VMAT-2). It has been shown that expression of VMAT-2 was decreased in isolated enterochromaffin-like cells that were pretreated with IL-1 and TNF-α [232]. Cytokines control dopamine release indirectly via stimulation of glutamate release from astrocytes [233]. In this manner, high levels of released glutamate from astrocytes stimulate production and release of dopamine that is by itself highly

susceptible to auto-oxidation, and these bio-products of dopamine oxidation lead to dopaminergic neuronal death [234].

A recent study of non-human primates has drawn attention to the fact that some midbrain dopamine neurons are not equally susceptible to CNS insults [235]. Dopamine neurons of SNpr are the most vulnerable one, whereas neurons of SNpc are less vulnerable and dopamine neurons of VTA are the most resistant to the neurodegenerative process [235].

Reactive microglial cells cause a negative impact on neuronal survival during inflammation. Upon treatment of isolated neuronal-glial cells with LPS, only mesencephalic (dopaminergic) neurons are shown to be more sensitive to degradation in comparison to hippocampal and cortical neurons [236]. Susceptibility of dopamine neurons to inflammation was in correlation with number of present ramified microglia cells [236]. Taking into an account that there is unequal distribution of microglial cells in the brain, being most abundant in an area of 5% in the cortex and corpus callosum to 12% in the substantia nigra [237], in inflammatory process the number of activated microglia was in correlation with extent of neuronal damage [236]. Another study in rodents showed that chronic intracerebral low doses of LPS injections selectively caused the loss of dopamine neurons in substantia nigra, while cholinergic neurons in the basal nucleus of Meynert and serotonergic neurons in dorsal raphe were unaffected [238].

1.9 Toxin-mediated degeneration of dopamine neurons

LPS has been recognized as a potent inducer of dopaminergic neurodegeneration and it exerts its neurotoxicity via activation of microglia cells [239]. In the CNS, LPS activates microglia cells as it binds to TLR4, which results in neuronal damage [240].

Besides LPS, there are several other toxins that have been described as potent inducers of dopamine neurons degeneration. A 6-hydroxydopamine (6-OHDA) is neurotoxin that shares structural similarities to dopamine and norepinephrine. It exhibits a deleterious effect on dopamine neurons via reactive oxidative species and quinones [241].

MPTP specifically induces dopamine cell death via its metabolite MPP (+) that accumulates in the dopamine cells causing the inhibition of ATP production and also induces the formation of free radical species [242].

Rotenone, mostly used as an effective pesticide and insecticide, causes the dopamine neurodegeneration by impairing the oxidative phosphorylation in mitochondria. It also inhibits the formation of microtubules from tubulin [243]. Tubulin was shown to be deleterious for cell in excess [244].

N,*N*′-dimethyl-4-4′-bipiridinium (paraquat), is a potent herbicide that exerts its deleterious effect through oxidative stress. It has been demonstrated that systemic injection of paraquat in mice resulted in reduced locomotor activity and loss of striatal dopamine fibers and dopamine neurons in substantia nigra [245].

1.10 Mechanisms of neuronal cell death of dopamine neurons

1.10.1 Oxidative stress

Oxidative stress is considered to be one major component associated with the development of neurodegenerative diseases. It occurs when free radical species (oxygen and nitrogen free radicals) are produced in excess and the anti-oxidative system is no longer capable of neutralizing these radicals. Free radical species originate from unpaired numbers of electrons presented on oxygen or nitrogen atoms. There are three major radicals generated from oxygen: superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$). Radicals generated from nitrogen are NO, and peroxynitrite (NO_3^-).

Free radical species are produced during many different metabolic processes but mainly in inflammatory states by infiltrating leukocytes at the site of the injury. Also their presence is relevant in processes such as cell proliferation, cell growth and glucose metabolism [246]. Besides the beneficial effect of free radical species, they also represent a constant danger to DNA, proteins and lipids of cell membrane. The free radical species interact with them in an unspecific manner, causing mutations, protein miss-folding, loss of enzymatic functionality and lipid peroxidations.

As the brain is one of the organs with the highest metabolic rate (20 % of total amount of oxygen) [247], it is more prone to oxidative stress. Neurons are shown to be more sensitive to oxidative damage due to high content of iron that can mediate

lipid oxidation of polyunsaturated fatty acids in the brain [247]. Moreover, neuronal cells are post-mitotic cells have higher lipid content and higher metabolic activity. At the same time the capacity of the antioxidative system is relatively low [248]. With aging, frequency of oxidative stress further increases as the process of antioxidant-mediated damage repair gradually slows down. Aging is considered one of the major factors implicated in the loss of dopamine neurons as it has been shown that 5-10% of their loss is linearly correlated with each decade of aging [249].

Dopamine neurons are susceptible to damage caused by ROS that are generated by the metabolism of dopamine itself [250]. Dopamine as a molecule is relatively unstable and prone to auto-oxidation, producing a high amount of ROS [250]. Its degradation may be spontaneous or mediated by monoamine oxidase B (MAO-B). Byproducts of dopamine metabolism are 3, 4- dihydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA) and small amounts of quinones, H_2O_2 and O_2^- [251]. Secreted dopamine is restored back into the cell by dopamine active transporter (DAT) that is shown to be depleted with aging, which results in the higher production of H_2O_2 and $^{\cdot}OH$ [252].

1.10.2 Mitochondrial dysfunction

Mitochondria are vital organelles responsible for the production of cellular energy in the form of ATP that is accomplished in the process of oxidative phosphorylation. On the other hand, this process is accompanied with the high production of free radical species that may constitute a potential danger for mitochondria functionality and cell survival overall. Alterations in mitochondrial complex I are considered to be the main reason for production of ROS [253].

Dysfunction of mitochondria caused by the large production of ROS is related with neuronal cell death [254]. In clinical studies, it has been reported that mitochondrial DNA was highly deleted in dopamine neurons of older in comparison to younger human brain tissues [255]. Deletion in of DNA in older human brain was associated with dysfunction of cytochrome *c* oxydase (complex IV) [255].

Impaired function of the mitochondrial permeability transition pore that is located in between the inner and outer membrane of mitochondrial cell membrane results in the release of cytochrome *c* in the cytosol, which in turn activates the caspase signaling pathway leading to programmed cell death [256].

1.10.3 Activation of the ceramide signaling pathway via TNF-α and IL-1β in dopamine cells

The TNF-ceramide signaling pathway is another and yet more specific mechanism of programmed cell death of dopamine neurons [257]. Ceramide belongs to a group of sphingolipids that has one fatty acid attached to its sphingoid base. Attached to a cellular membrane, ceramide is a constitutive component that contributes to increased membrane permeability [258]. Ceramide is also involved in a signaling pathway that controls cell proliferation and differentiation and also cell death [257].

TNF-α-induced dopaminergic cell death is triggered via TNFR1 [259] followed by the activation of the ceramide signaling pathway. TNFR1 is expressed on the cell membrane of dopamine neurons and is involved in the transmission of cell death signaling [260]. Having established a connection to TNFR1, TNF-α activates membrane-bound sphingomyelinases resulting in the conversion of sphingomyelin into ceramide *in vitro* [257]. With the activation of these signaling pathways, ceramide-induced cell death follow several different routes of actions: increase in intracellular Ca^{2+} [261], mitochondrial dysfunction [262], oxidative stress in endoplasmic reticulum [263], inhibition of survival pathway (PI3K/Akt) [264] and promotion of caspase-3 dependent apopototic process [257]. Similarly, the sphingomyelinase death-signaling pathway has been triggered by activation of the IL-1β pathway [140, 265].

1.10.4 Inflammasome assembly and release of proinflammatory factors (activation of caspase-1 through the TLR4 signaling pathway)

The inflammasome represents an intracellular multiprotein complex that mediates the activation of caspase-1, which in turn cleaves proinflammatory cytokines (IL-1β, IL-18 and possible IL-33) into their mature form [266]. A key component of the inflammasome is PYD domains-containing protein 3 (NALP3) also known as cryopirin or NLRP3, the name of which represents also the name of the protein complex. It belongs to the NLR (nucleotide-binding oligomerization domain-like receptor) family of proteins involved in the regulation of the innate immune response, and it is expressed in microglia [267].

The structure of NALP3 is composed out of three different domains: PAMP/DAMP ("pathogen-associated molecular patterns"/ "danger-associated molecular patterns")-sensing C-terminal leucine-rich repeat (LRR), a central nucleotide binding (NACHT)

domain and an N-terminal effector domain, namely pyrin domain (PYD) [113] (figure 1.6). Activation of NALP3 inflammasome may be induced by membrane damage in the presence of several insults: 1) extracellular ATP originating from dying and injured cells activates NALP3 through P2X7 receptors and subsequent release of 2) intracellular potassium [268] and 3) crystals such as uric acid and calcium pyrophosphatedihydrate. Along with the P2X7 receptor, another type of channel namely pannexin-1, has been reported to play a role in ATP-induced NALP3 activation [269]. Other relevant danger signals that lead to the activation of the NALP3 inflammasome include the recognition of PAMPs through TLR4. Under basal conditions in the brain, TLR4 is found in the choroid plexus, leptomeninges and CVO`s in the membrane of resident immune cells [270].

Upon activation, the PYD domain, one of the NALP3 domains interacts with an adaptor molecule speck-like protein containing a caspase recruitment domain (ASC). After being translated, caspase-1 exists in the form of inactive zymogen (pro-caspase 1). Pro-caspase-1 carries its activation through domain, namely caspase activation and recruitment domain (CARD), which binds with the CARD domain present on ASC. Pro-caspase-1 is then processed into its active form: caspase-1 [113] by proteolysis [271]. Activated caspase-1 cleaves the immature forms of IL-1β [272], and IL-18 [273], which are immediately released from the cells [274] (figure 1.6).

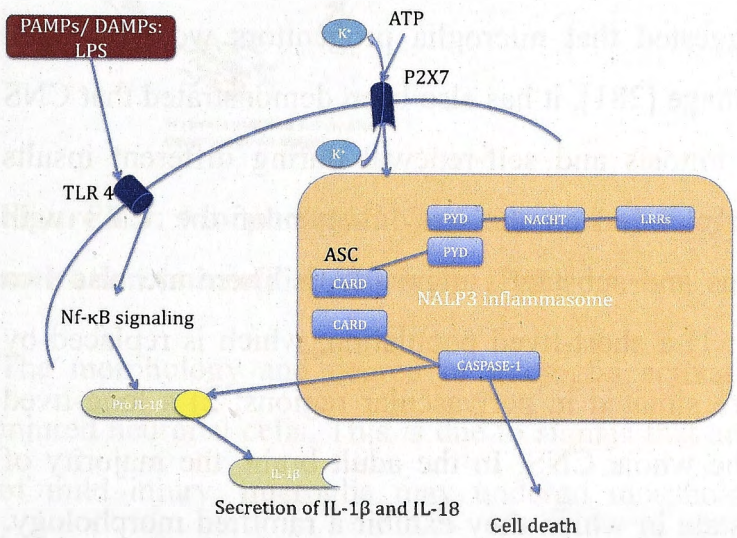


Figure 1.6: Structural representation of the NALP3 inflammasome and the possible signals required for its activation.

1.10.4.1 TLR- mediated tolerance to the inflammatory process (tolerance to repeated administration of LPS)

Upon LPS stimulation activated TLR4 mediates the production of various cytokines, chemokines, prostaglandins and NO from resident immune cells in the brain. Frequent exposures to LPS injections have been shown to significantly increase production of proinflammatory cytokines in comparison to only one injection [275].

Even though repeated systemic inflammation may subsides with increased tolerance to cytokine production, a similar outcome is not necessarily seen in the brain. For instance, during systemic LPS-induced inflammation, expression of cytokines in the brain was reported to be quite high while in the periphery the cytokines were no longer detected [276]. Similarly, it was concluded that resident immune cells in the brain are not tolerant of peripheral inflammation, instead these cells become primed, which may lead to prolonged production of cytokines and progression of disease [277].

1.10.5 Role of microglia activation in the degeneration of dopamine neurons

In the 1930`s, Pio del Hortega estimated that microglia cells accounts for approximately 12% of the brain [278]. Microglia are resident immune cells of the CNS that have major roles in monitoring the brain for any kind of insults and invading pathogens [279]. In the past, it was thought that microglia cells originated from circulating blood monocytes that reached the brain parenchyma [280]. Although previous evidence suggested that microglia progenitors were recruited from the periphery upon CNS damage [281], it has also been demonstrated that CNS resident microglia can undergo mitosis and self-renewal during different insults [282]. Microglia cells are mostly found in the grey matter of the CNS with predominance in the hippocampus and substantia nigra [283]. There are also two distinct populations of microglia: 1) a short-lived population, which is replaced by the circulating monocytes and are situated in perivascular regions; 2) a long-lived group of microglia residing in the whole CNS. In the adult brain, the majority of microglia remain in a "resting" state in which they exhibit a ramified morphology. Microglia cells in the "resting" state are characterized by small cell bodies and many small branches, containing up to 20 spines. On the other hand, activated microglia display ameboid cell shape with truncated branches. Resting microglia are not static, dormant cells. Rather, they are constantly monitoring the area in which they reside.

In other studies it has been suggested that microglia cell bodies do not move during normal surveillance, but rapidly and dynamically extend and retract their processes [284, 285].

There is also a third state of microglia activation, namely the hyper-ramified state, which represents an intermediate stage between the resting and the reactive forms (figure 1.7). Hyper-ramification represents the beginning of microglial hypertrophy. Even though this state is rarely seen, it occurs during aging and under certain degenerative changes [286]. In late life, microglia hyperthrophy may become extreme; they tend to lose contact inhibition and begin to fuse with each other. Such fusion can result in the formation of small microglial clusters, which are suspected to be responsible for the formation of senile plaque [287].

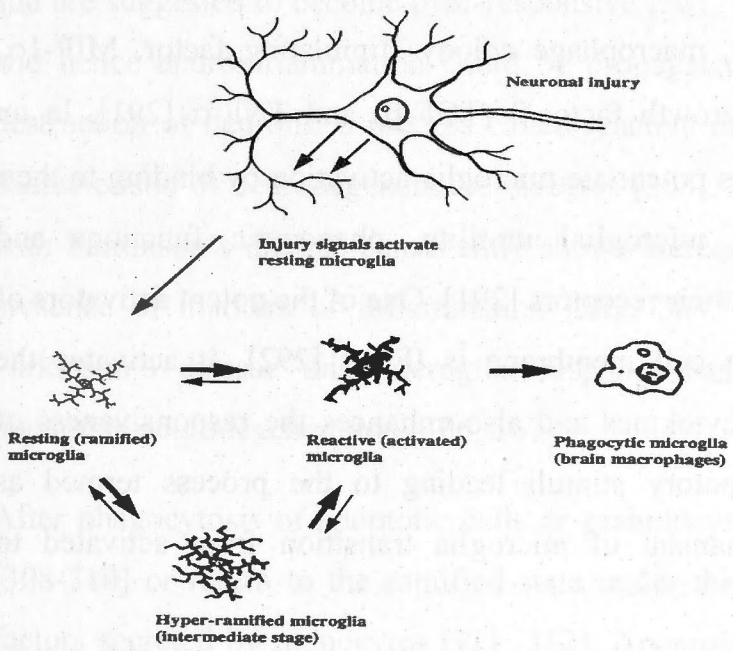


Figure 1.7: Morphological presentation of microglial cells in different stage of activation (microglial plasticity) [288].

The morphology and degree of microglia activation depends on the severity of injured neuronal cells. This is due to signals that are emitted by dying cells. In case of mild injury, microglia may undergo morphological changes turning into the hyper-ramified state. In most cases, microglia becomes activated to remove dying cells. However, if injured cells start to recover, then microglia might revert to the ramified state (resting microglia). If microglia have already undergone transformation into macrophages, then they are predestined for cell death [288].

1.10.5.1 Microglia activation

Microglia are capable of recognizing a vast variety of insults within the CNS. They display a battery of cell-surface receptors, including receptors for endotoxin, cytokines, chemokines, mis-folded proteins, serum factors and ATP. Microglia also act as antigen-presenting cells (APc) and present pathogenic material to T-cells to generate an adaptive immune response through expression of major histocompatibility complex (MHC) class II antigen [283, 289].

A widely used experimental approach for *in vitro* and *in vivo* activation of microglia is the administration of LPS. As it was mentioned earlier LPS activates microglia by interacting with TLR4 [60]. It has been reported that microglial cells display 9 of 12 members of TLRs [290]. LPS-induced microglia activation results in the synthesis and release of a number of cytokines and chemokines such as IL-1β, IL-1α, IL-1ra, IL-6, IL-8, IL-10, IL-12, IL-18, macrophage colony stimulating factor, MIP-1α, MIP-1β, MCP-1, transforming growth factor-β (TGF-β) and TNF-α [291]. In an autocrine manner, these cytokines potentiate microglia activation by binding to their receptors [291]. They control microglial motility, phagocytic functions and chemotactic movements through their receptors [291]. One of the potent activators of cytokine receptors on microglia cell membrane is IFN-γ [292]. It activates the expression of proinflammatory cytokines and also enhances the responsiveness of microglia to the other inflammatory stimuli leading to the process termed as "priming". The complete mechanism of microglia transition from activated to ramified is still unsolved [292].

Microglial cells, are also activated by IL-1, and under the influence of pathological concentrations (nM) can synthesize a variety of inflammatory mediators such as adhesion molecules [293], chemokines [294] and prostaglandins [295]. Two other potential activators of microglia are matrix metalloproteinase-3 (MMP-3), and neuromelanin [296, 297]. It is also been suggested that activation of microglia may occur due to a "switching-off" mechanism of the inhibitory effect that neurons exert on microglia during baseline conditions. In this case, expressed neuronal CD200, a cell-surface transmembrane glycoprotein, binds to microglial CD200R [298], maintaining the microglial cells in a quiescent state in the healthy brain [291]. There are multiple factors that may contribute to the transformation of microglia to the resting cell phenotype, such as TGF-β and IL-10 [299]. Most recently a group of

nuclear receptors of transcription factors were discovered, which might suppress the microglial activated state: estrogen receptor-α (ER-α), peroxisome proliferator-activated receptor-γ (PPARγ), liver X receptor-α (LXRα) and LXRβ have been described to attenuate the symptoms of Parkinson`s disease [300].

Activated microglia is considered to be vital for normal brain function because they have beneficial effects by acting as scavengers and by remodeling brain tissue. On the other hand, continuously activated or over-activated microglia can cause viable cell damage, particularly neurons. The activation of microglia caused by toxins, pathogens or endogenous protein, might persist for a long-term due to positive feedback of damaged neurons, even if the initial insult has ceased. During the aging process chronically primed microglia may release more proinflammatory cytokines and are suggested to become over-responsive [301, 302]. Thus, microglia activation, and hence neuroinflammation could be propagated and prolonged to amplify the destruction of neurons: a process called reactive microgliosis, which is a common characteristic of neurodegenerative diseases [303]. The ventral midbrain of patients with Parkinson's disease consistently shows increased microglial reactivity and the presence of markers of inflammation [283, 304, 305] and in animal models of Parkinson's disease this microglial response has been shown to contribute to dopamine neurodegeneration [306, 307].

After phagocytosis of apoptotic cells or granulocytes, microglia undergo apoptosis [308-310] or return to the ramified state under the influence of anti-inflammatory factors secreted by monocytes [311, 312]. Apoptosis is carried out by the caspase-dependent pathway or by another caspase-independent programmed cell death [313].

1.10.5.2 NF-κB activation and cytokine expression in microglia

The inflammatory response begins with recognition of LPS by TLR4 on microglia membrane. Apart from binding TLR4, LPS is also recognized by LPS-binding protein, CD14 (protein which mediates LPS recognition) and MD-2 (a soluble protein that can form a complex with LPS) [314, 315]. LPS may be also recognized by the presence of P2X7 receptors that reside on the cell membrane.

Immediately after interacting with LPS, the intracellular domain of TLR4 namely, Toll-interleukin-1 (TIR) associates with MyD88, a TIR domain-containing adaptor. By recruiting the TLR4 receptor and TIR assembly, the MyD88 downstream

signaling pathway continues in two different directions: 1) the MyD88-independent pathway, which mediates induction of the Type I interferon and 2) MyD88-dependent pathway that is responsible for the expression of proinflammatory cytokines. The MyD88-independent signaling pathway is particularly associated with TLR4 signal transduction [316]. Upon activation of the MyD88, it recruits interleukin-1 receptor associated kinase 4 (IRAK4), which in turns phosphorilates and activates IRAK1. Then, the activated IRAK1 associates with tumor necrosis factor receptor-associated factor 6 (TRAF6). TRAF6 is responsible for further activation of transforming grow factor-β-activated protein kinase 1 (TAK 1) which undergoes ubiquitination and forms a complex with TAB1, TAB2 and TAB3. Once activated, TAK1 recruits the I kappa B kinase (IKK) complex and catalyzes the phosphorilation of two N-terminal serine residue on I-κB. These two phosphorylated serines constitute a target for the E3 ubiquitin ligase, which polyubiqutinates I-κB. Thereafter, I-κB undergoes degradation releasing NF-κB. Afterwards, NF-κB is translocated into the nucleus and binds to DNA on specific NF-κB binding sites that are present on the promoter of inflammatory cytokines. At the same time, TAK1 also activates mitogen-activated protein kinase (MAPK) that in turn activates activator protein-1 (AP-1), one of the transcription factors that also controls the expression of proinflammatory cytokines (figure 1.8).

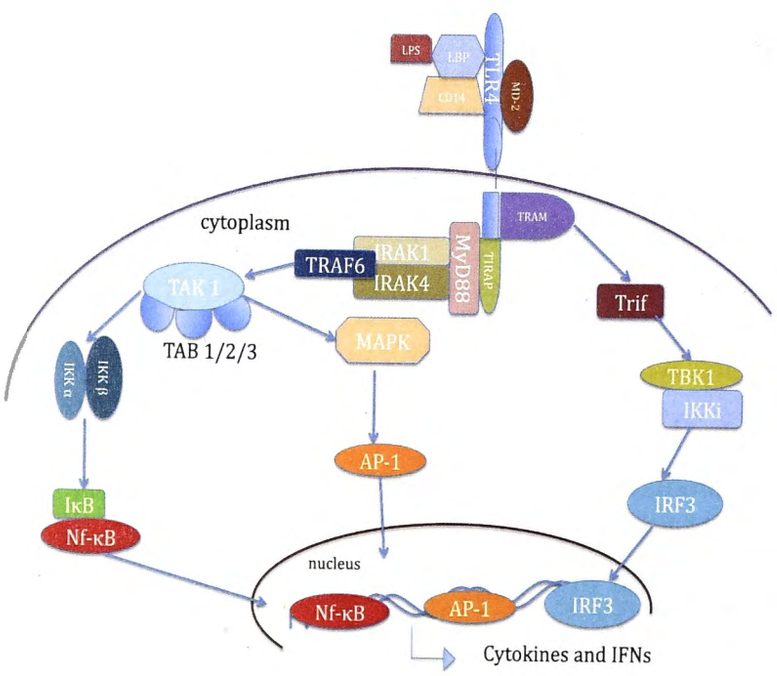


Figure 1.8: TLR-4 mediated signaling pathway.

The MyD88-independent pathway is followed by the recruitment of TRAM and Trif, which interacts with TANK-binding kinase 1 (TBK1). TBK1 and IKKi (IκB kinase) conduct the phosphorylation of interferon regulatory factor 3 (IRF3), which forms dimers and translocates into the nucleus to bind to the DNA (figure 1.8) [316].

1.11 Obesity and its effect on dopamine system

1.11.1 High-fat diet-induced obesity

Term obesity is referred to a state of the increased accumulation of lipids into the adipose tissue. The level of fat deposition depends not only on general food intake, but also on basal metabolic rate, body thermoregulation and physical activity. In particular, increased intake of food rich in fat has been associated with weight gain and development of obesity in humans [317]. Currently, the percentage of obesity in the global human population has reached epidemic scale. According to a report of WHO (World Health Organisation), 10% of the male and 14% of the female global population are obese [318]. Obesity increases the risk of the development of several metabolic disorders such as type-2 diabetes, glucose intolerance and insulin resistance, hypertension and dyslipidemia [319].

Diet-induced obesity in rodents is achieved by introducing food rich in carbohydrate and fat with the purpose of mimicking obesity in population of humans that are exposed to a Western diet on a daily basis. The first model of diet-induced obesity was introduced in 1955, where substantial weight gain reported in rats was due to the excess consumption of food rich in fat [320]. Feeding the animals with such high calorie diet results in significant weight gain with concomitant occurrence of various metabolic problems seen in obese human population. For instance, the first parameter observed in animals fed a high-fat diet is that these animals tend to consume more calories than the controlled group [321]. This condition is known as hyperphagia, the term that is described as the inability of an individual to regulate its own food intake. High-fat diet consumption also has a role in remodeling the fat tissue by increasing the number (hyperplasia) and the size (hypertrophy) of adipocytes [322]. Other metabolic alterations caused by high-fat diet induced obesity involves the occurrence of insulin resistance [323], increased lipogenesis, increased intestinal capacity for fat absorbtion [324] development of hepatic steatosis [325] etc.

1.11.2 Obesity and inflammation

Obesity has been recognized as a state of a low-grade chronic inflammation [326]. Inflammation in obesity is caused by the excess intake of nutrients, which as a consequence leads to increased metabolic rate in adipocytes. Therefore, it has been hypothesized that nutrient overload in adipose tissue induces intracellular stress, which further contributes to the development of local inflammation [327].

Even though it was considered that the sole function of adipocytes is in storage of excess energy, discoveries made in past decade led to the conclusion that adipose tissue may operate as an endocrine organ as well [328]. The first hormone that was found in adipocytes is leptin, which functions to down-regulate food intake and increase energy expenditure [328, 329]. Adiponektin is another hormone secreted from adipocytes that was shown to negatively correlate with body weight and the dysregulation of which was associated with the development of metabolic syndrome [330]. The third hormone produced in adipocytes is resistin that was shown to down-regulate insulin activity [331].

The condition of excessive food intake and fat accumulation triggers hyperactive adipocytes to release MCP-1 that further stimulates infiltration of macrophages into the adipose tissue [332]. There are two types of macrophages that may be found in adipocytes: 1) The M1 cell population is group of activated macrophages in their proinflammatory state and 2) The M2 is an anti-inflammatory cell population. In obese mice this M1 cell population was shown to be prevalent and had the negative impact of insulin sensitivity [333]. Infiltration of the immune cells in adipose tissue happens gradually forming aggregates known as Crown-like structures [334]. This macrophage infiltration is accomplished with the presence of not only MCP-1 but also of chemokine (C-C motif) receptor 2 (CCR2), as the knockout mice for these genes did not show macrophage infiltration [332]. Besides macrophages, in adipose tissues the presence of circulating lymphocytes [335], mast and natural killer T cells [336] was also reported. Activation of immune cells in adipose tissue is followed by the release of inflammatory markers such as IL-1β, IL-6 and TNF-α [337]. The prolonged inflammatory process then alters the proper function of immune cell into a proinflammatory environment. Such prolonged obesity-induced inflammation becomes chronic and unresolvable.

A diet rich in fat and carbohydrates may also influence the composition of gut microbiota and therefore contribute to greater weight gain [338] and enhance permeability of gut to inflammatory components, such as LPS. Microbes that are hosted in the gut of animals and humans are collectively named microbiota and their function is to improve digestion and in aid in the synthesis of some essential compounds (vitamins). Change in diet may profoundly influence the ratio between populations of different species of microbes which in turn stimulates weight gain as it was shown that microbita from obese animals had a higher capacity to harvest more energy in comparison to group of animals that were on a standard chow diet [339, 340]. Excessive food intake high in calories has been shown to induce an inflammatory state as mice that were chronically infused with lower dose of LPS had similar phenotypic characteristics with mice fed a high-fat diet (increased body weight, inflammation and hyperglycaemia) [341]. Moreover, it has been observed that gut microbiota in animals fed a high-fat diet produces substantial amount of LPS that is further transduced into circulation, which is considered to partially contribute to various metabolic complications seen in obesity [342].

1.11.3 Effect of high-fat diet and metabolic outcomes on the depletion of dopamine neurons

Inflammation that originates from the adipose tissue may also influence the functionality of the brain. Insulin resistance, induced by the consumption of high-fat diet is accompanied by weight gain, increased stress and hyperglycemia. These metabolic outcomes could make dopamine neurons more susceptible to the environmental insults. For example, adiposity [343] and type-2 diabetes [344] were already suggested to be linked with Parkinson`s disease, as it was reported that 50% of Parkinson`s diseased patients had an abnormal glucose tolerance [345] or diabetes [346]. Also epidemiological studies have shown that there could be a connection between consummation of animal meat rich in fat and development of Parkinson`s disease [347]. Brain fatty acid composition is also changed with high-fat consumption. Nine months of high-fat diet consumption causes alteration in brain fatty acids, such as a decreased ratio of n-3 and n-6 PUFA (omega-6 polyunsaturated fatty acids) [348]. One of the member of 6-PUFAs, arachidonic acid was noticed to be a contributor to the proinflammatory environment via cyclooxygenase and lypoxygenase action producing several bioactive eicosanoids [349]. Eicosanoids products were reported to be present in Parkinson`s disease [350].

In animal studies, a high-fat diet consumption leads to the development of systemic inflammation characterized by increased plasma level of leukocytes, granulocytes, B and T lymphocytes [351], and group of three cytokines: MCP-1, IL-1α and MIP-1α [351]. In addition, it has been shown that expression of inflammatory cytokines (e.g. TNF-α, IL-6 and IL-1β) was increased in the hypothalamus of obese animals and were related with undergoing apoptotic process of the hypothalamic neurons [352]. In the presence of such high systematic inflammation, obese animals are shown to be more susceptible to a greater depletion of nigrostriatal dopamine neurons than the animals fed a chow diet, as observed through the intranigral infusion of MPTP [351] or 6-OHDA injection [353].

1.11.4 Effect of leptin on levels of dopamine and dopamine receptors

Leptin is a 16kDa peptide secreted by the adipose tissue. Its primary function is to control food intake. Its plasma levels are proportional to total body fat [354]. Leptin is transported through the BBB by binding to the leptin receptor, present in the cerebral capillaries [355]. Food intake causes an increase in leptin secretion, which in turn activates leptin receptors in the brain to inhibit appetite and stimulate thyroid-regulated thermogenesis and oxidation of free fatty acids. More specifically this process of leptin signaling in the brain is established in arcuate nucleus of the hypothalamus [356]. It has been shown that leptin transport and function in CNS is impaired in obese patients and rodents [357]. This functional dysregulation of leptin has been shown to negatively affect bone formation, cause cardiovascular and reproductive problems [357] and weight gain. Leptin also has a role in immunomodulation by increasing the phagocytic function of immune cells and production of proinflammatory cytokines [358].

Leptin is positive regulator of dopamine release. Immunohistochemical analysis showed that leptin receptors are expressed within dopamine neurons of VTA and substantia nigra [359]. It has been suggested that the function of leptin and also the insulin receptor is in glucoregulation [360]. The function of dopamine neurons in the release of dopamine within SNpc to striatum has been observed to be dependent on ATP-sensitive K^+ channels activity, which is directly related with the level of glucose [361]. In connection with the previous statement, leptin was shown to be involved in the regulation of potassium channels activity in the hypothalamus [362] and therefore it is believed that in a similar manner leptin may regulate dopamine

release in SNpc. In support of that notion is the finding that leptin receptors are co-expressed with tyrosine-hydroxylase in areas of VTA and substantia nigra [363]. Leptin has been shown to be protective against the loss of dopamine neurons in 6-OHDA of Parkinson`s disease, both in vitro and in vivo [364]. For instance, treated dopaminergic cells with 6-OHDA had a greater survival rate in cases where leptin was present [364].

As leptin may be implicated in the control of dopamine release it is also suggested that it has a role in dopamine storage, since leptin-deficient (*ob/ob)* mice have been reported with decreased dopamine stores in dopamine neurons of the midbrain [11]. Studies conducted in rats showed that there might be direct correlation between the decrease of dopamine and high levels of leptin [365]. In regard to the previous statement, a study conducted on animals fed a high-fat diet showed that high levels of leptin might be implicated in down-regulation of TH enzyme expression [366].

Leptin also regulates the availability of the dopamine D2 receptor. In the cases of severe obesity, which is followed by leptin and insulin insensitivity, striatal dopaminergic activity may stay unchanged and in turn cause compensatory down-regulation of D2 receptors [367]. It has been found that in obese individuals levels of D2 receptors are decreased [367]. Similarly, the decline of D2 receptors was also observed in the striatum of leptin-deficient obese rodents [368]. Another study reported that administration of D2 receptor agonist decreased food intake, increased locomotor activity and D2 receptor binding in leptin-receptor deficient obese rats [369].

1.11.5 Effect of insulin resistance on dopamine neurons

The function of insulin is to mediate glucose clearance from peripheral circulation into skeletal muscles and adipose tissues [370]. This is accomplished by the presence of insulin receptor IRS (insulin receptor substrate). Upon insulin binding to its receptor, glucose transporter type 4 (GLUT-4) is translocated from intracelular storage to the cell membrane, which then enables glucose transport into the cell [371]. The process of glucose transport may be disrupted if specific serine residue of IRS-1 is phosphorylated as this interferes with correct association to insulin receptor [372].

Inflammatory cytokines released from infiltrating macrophages in adipose tissue negatively affect insulin sensitivity. It is shown that elevated TNF-α and free fatty acids may activate serine kinase, which in turn phosphorylates serine residue of IRS-1 and hence blocks insulin signaling [373]. The inflammation created in the state of obesity is sensed by the activation of two stress sensors (serine kinases- JNK and IKK) that have been shown to be also linked with insulin resistance [374]. As an example of insulin sensitivity dysregulation in the presence of proinflammatory cytokines, adipocytes that were treated with TNF-α showed to have decreased glucose intake and insulin signaling [375]. Dysregulation in insulin signaling in animals fed a high-fat diet showed also to have a negative impact on dopamine release followed by a decrease in dopamine clearence [12].

Overall, the inflammatory state of obesity that develops as a consequence of high-fat consumption and metabolic disorders such as insulin resistance cumulatively elicts a negative effect on the functionality of dopamine neurons and their survival.

1.12 Research questions

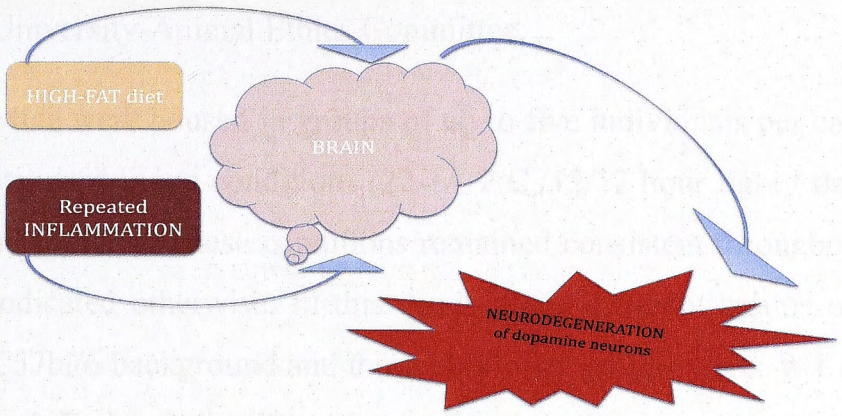
The purpose of this project was to explore the possible connection between inflammation, elicited by repeated sublethal dose of LPS and increased caloric intake, on the onset and progression of dopaminegic neurodegeneration in a time- and dose-dependent manner. Although mounting evidence suggests that neuroinflammation plays a pivotal role in neurodegenerative diseases, there is still little understanding about the key role that prototypic proinflammatory factors such as IL-1β play in neurodegeneration. Moreover, it is not well understood weather environmental factors such as increased energy intake leading to excess weight and/or obesity could elicit neuroinflammation and/or neurodegeneration. Therefore, there are two questions to be answered:

What is the possible role of the IL-1-related pathway in inflammation-induced dopaminergic neurodegeneration?

Is there an exacerbation in inflammation-induced dopaminergic neurodegeneration caused by increased caloric intake?

1.13 Hypothesis

LPS inflammatory challenge and **increased caloric intake, caused by exposure to high-fat diet, will cause microglial activation and dopaminergic neurodegeneration through a process involving the IL-1 pathway.**



Experimental aims:

Hypothesis 1) Systemic inflammation induces dopaminergic degeneration followed by activation of the IL-1 pathway in resident microglial cells of CNS.

AIM 1: To study the long-term effect of systemic LPS challenge on behavioral, metabolic and inflammatory outcomes, exerted through the IL-1 pathway.

Hypothesis 2) LPS inflammatory challenge may be exacerbated by the high-fat diet exposure.

AIM 2: To study the long-term effects of high-fat diet consumption and systemic LPS challenge on behavioral, metabolic and inflammatory outcomes.

To investigate these two major hypothesis, the experiments were designed based on the previous work reported by a research team from North Carolina [376] with the addition of Casp-1-/-, IL-1ra-/- and WT mice fed a high-fat diet and with the implementation of the other behavioral tests to explore more thoroughly long-term effect of LPS inflammatory challenge.

2. Materials and methods

2.1 Experimental Plan and Methodology

All animal experiments were performed according to the rules and regulations of the "Australian code of practice for the care and use of animals for scientific purposes"; therefore, all the experimental protocols were approved by the Australian National University-Animal Ethics Committee.

Mice were housed in groups of up to five individuals per cage and maintained under standard living conditions (22 +/- 2°C, 12/12 hour light / dark cycle, food and water ad libitum). These conditions remained consistent throughout the experiment unless indicated otherwise. In this thesis, three different strains of mice were bred in the C57bl/6 background and those employed groups were: WT (n=60), Casp-1-/- (n=30) and IL-1ra-/- (n=30) mice. All mice were randomly assigned to the different experimental groups. The WT mice were divided into two groups (n=30) and were fed either a regular chow diet (RD) or a high-fat diet (HFD). The Casp-1-/- and IL-1ra-/- mice that were used in this thesis were previously described by Li, P et al [377] and Hirsch, E et al [378], respectively. All knockout mice were fed a regular chow diet. Mice from each group (genotype and diet) were divided into 3 groups:

- Group 1: injected five times with saline - one injection per month [10 mice from WT (fed either a RD or HFD), Casp-1-/- and IL-1ra-/- genotype].

- Group 2: injected only once with LPS and 4 times with saline - one injection per month [10 mice from WT (fed either a RD or HFD), Casp-1-/- and IL-1ra-/- genotype].

- Group 3: injected five times with LPS - one injection per month [10 mice from WT (fed either a RD or HFD), Casp-1-/- and IL-1ra-/- genotype] (table 2.1).

Casp-1-/- and IL-1ra-/- mice were not subjected to high-fat diet since the workload with the current mice groups was extensive and it involved long-term behavioral assessment over thirteen months period. Due to these factors, the preference was drawn to high-fat diet-fed WT mice in order to expand the research based on the previous published study [376].

Diet	Treatment	WT	Casp-1-/-	IL-1ra-/-
Regular diet	Saline (control)	10	10	10
	LPS (1 time)	10	10	10
	LPS (5 times)	10	10	10
High-fat diet	Saline (control)	10	-	-
	LPS (1 time)	10	-	-
	LPS (5 times)	10	-	-

Table 2.1: Schematic representation of the experimental groups employed in this thesis: Mice were 47 days old when they received their first saline/LPS injection. All mice were injected 5-times per month. Mice from the control group received 5 injections of saline. Mice from the LPS 1-time groups received the first LPS injection when they were 47 days old, and 4 saline injections per month. Mice from LPS 5-times group received five LPS injections per month.

2.2.1 Genotyping of mice

Casp-1-/- mice were bred in a homozygous state, whereas IL-1ra-/- mice were bred in a heterozygous state. Thus, the genotype of IL-1ra-/- mice was determined by PCR. For this purpose, DNA was extracted from ear punches at weaning with DNA extraction kit (Life Technologies). The concentration and quality of purified DNA was determined by spectrophotometry by using a NanoDrop apparatus (Spectrophotometers, Thermoscientific). In the IL-1ra-/- mice, all four isoforms of IL-1ra were disrupted [378]. Since IL-1ra-/- mice were generated by inserting neomycin resistance gene (NEO) within the *IL-1ra* gene, primers were synthesized as one set specific for T14/T16 loci (T14/T16 OX) and another set specific for NEO to differentiate homozygous and heterozygous [378].

T14/T16 OX primers:

Primer F (OX)- 5'- TGGAGCTCTGGTTCACTGTA-3'

Primer R (OX)- 5'- ATCCTGGACAGGCAGCTGACTC-3'

The PCR reaction was performed as follows: 1) 94°C for 3 minutes, 2) 94°C for 30 seconds, 3) 64°C for 30 seconds, 4) 68°C for 2.5 minutes; steps 2 to 4 were repeated in 34 cycles, 5) 68°C for 7minutes and 6) 10°C ∞.

NEO primers:

Primer F (OX)-5'- AGACAATCGGCTGCTCGAT-3'

Primer R (OX)-5'- CAATAGCAGCCAGTCCCTTC-3'

The PCR reaction was performed as follows: 1) 94°C for 3 minutes, 2) 94°C for 30 seconds, 3) 58°C for 30 seconds, 4) 68°C for 1 minute; steps 2 to 4 were repeated in 34 cycles, 5) 68°C for 7minutes and 6) 4°C ∞.

The PCR reactions were performed in an Eppendorf Termocycler and the products of amplification were analyzed by agarose gel electrophoresis.

2.2.2 Intraperitoneal injection of LPS

Animals were injected intraperitoneally with 0.9% NaCl (saline), or 5 mg/kg of lipopolysaccharide from *Escherichia coli* (strain O111:B4, Sigma-Aldrich), previously dissolved in saline. The dissolved drug was filtered, aliquoted and stored at -20°C until usage. Mice were injected with the first saline/LPS injection when they reached 47 days of age.

2.3 Behavioral tests

Two behavioral tests namely, rotarod and open-field were performed to ascertain locomotor skills throughout a period of nine months starting before (baseline) and after (nine months) from the first saline/LPS injection:

1) Rotarod test: it was conducted twice a month [the first week (seven days after saline/LPS injection) and in the last week].

2) Open-field test: it was performed the third and last week after saline/LPS injection.

This time period was chosen due to previous research results that showed that the first significant difference in behavioral tests, between control group and five monthly LPS-injected WT female mice, was observed after 7 months (in rotarod test) and 9 months (in open-field test) [376].

2.3.1 Rotarod test

This test is used to measure locomotor ability (balance and coordination) of mice to stay on the rotating drum of the rotarod apparatus (Panlab, Harvard apparatus, Spain, Barcelona) (figure 2.1). The rotarod starts at a baseline speed of 4 revolutions per minute (rpm) and accelerates to 40 rpm within the timeframe of 2 minutes. Mice were given 3 trials with 2 minutes break between trials. Prior to starting data collection, mice were trained for 4-6 days to achieve optimal performance in the rotarod test. The training started when mice reached the age of 43 days. During the training period all mice were fed a regular chow diet and were not injected. The latency to fall from the rotating drum was measured in seconds. Mice that show better balance and coordinative skills are able to remain for a longer period of time on the rotating drum. The final result was calculated as the mean of the latency to fall from the rotarod apparatus after 3 trials. In the infrequent event when mouse did not fall from the rotating drum after 2 minutes, it was removed from the apparatus and returned to its home cage [376]. As it was previously shown, five LPS injections (i.p.) induced the locomotor impairment in female mice that was observed in the rotarod test, seven months post initial LPS injection [376].

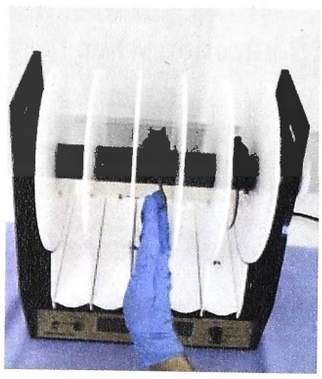


Figure 2.1: Rotarod apparatus. Five mice were individually placed on the rotating drum of the rotarod apparatus at initial speed of 4 rpm. Afterwards, accelerator mode (4-40 rpm within 2 minutes) on the apparatus was initiated and latency of each mouse to fall from the rotating drum was recorded in seconds.

2.3.2 Open-field test

The open-field test was used for measuring locomotor activity, explorative and anxiety/depressive-like behavior. This test was conducted by placing the mice separately in individual arenas (figure 2.2). The locomotor activity was recorded by camera in time frame of 32 minutes. The baseline performance of mice in the open-field test was recorder when they reached age of 42 days. The data were collected and processed by the software "Viewer 3" (Biobserve, Germany

www.biobserve.com). The aim of this test was to measure the total distance travelled in the area of the arena where the mice moved. Rodents tend to avoid open bright areas. Consequently, those that move more in the centre of the arena are considered to display less anxiety/depressive-like behavior. Thus, two key end-points were determined in this behavioral test: 1) Total traveled distance (measured in cms), which reflected their locomotor activity [376] and 2) The ratio between central distance over total distance (CD/TD), which was used as an index of anxiety-like behavior in the final analysis. As mentioned above, the more time each mouse spends in the center of the arena, the less anxious they are. In the figure 2.2 a picture of 4 arenas are shown. Each arena has a red square in the center depicting the central area considered for the calculus of the center distance travelled by the time.

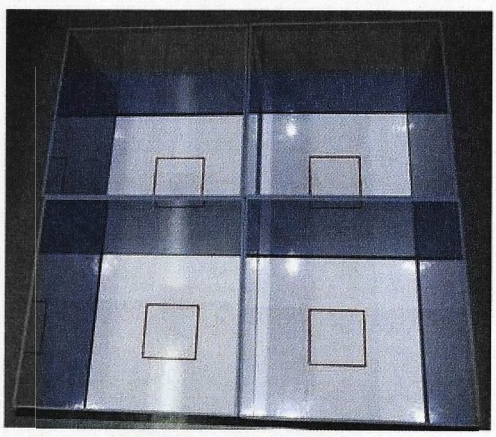


Figure 2.2: Open-field arena. Each mouse is individually placed in the corner of the open-field box and animal behavior is recorder in next 32 minutes by the software ("Viewer 3").

Since dysregulation in dopamine system may be accompanied by others, non-motor symptoms such as anxiety, depression and cognitive impairment, at the end of the nine months, following tests were conducted:

- Tests for evaluation of anxiety/depressive-like behavior: 1) Elevated plus maze test, 2) Forced swimming test and 3) Novelty suppressed feeding test.

- Tests for evaluation of cognitive function: 1) Prepulse inhibition test and 2) Morris water maze test.

Locomotor abilities in mice were further evaluated in the following tests: 1) Grid test, 2) Pole test, 3) Stepping test and 4) Hind limb clasping test.

All the tests listed above were conducted over a period of 9 to 13 months post first saline/LPS injection. This time frame was chosen since it was previously reported that significant LPS-induced coordinative and locomotor disabilities in mice observed in a period of 7 to 9 months, were related with dopaminergic neurodegeneration [376].

2.3.3 Elevated plus maze test

The elevated plus maze apparatus was used to assess anxiety-like behavior. The maze was elevated 100 cm above the floor and has closed and an open arm (40 cm length x 8 cm width). The wall of the closed arm is 18 cm high and the open arm is surrounded with the rim (0.5 cm high) to prevent animals from falling (figure 2.3).

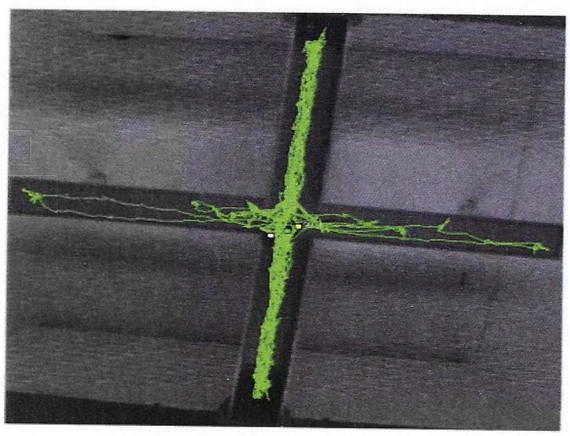


Figure 2.3: Elevated plus maze apparatus

The test is performed by placing the animal in the intersection of two arms, facing it towards the open arm (figure 2.3). The animal activity is recorded over a period of 5 minutes with a camera coupled with software "Viewer 3" (Biobserve, Germany www.biobserve.com). The proportion of time (in seconds) that each mouse spends in the open and closed arm is used as the parameters in the final analysis. Less anxious animals will spend more time in an open arm and conversely less in a closed arm. In contrast, more anxious animals will spend less time in an open and more in a closed arm. As mentioned before, the experiment is conducted during the dark phase, as the animals are more active during nocturnal hours [379].

2.3.4 Prepulse inhibition test

The prepulse inhibition test aims at measuring a neurological outcome in which a weaker acoustic stimulus (prepulse: few decibels above the background noise) inhibits the reaction of an organism to "startle" after being exposed to a subsequent

very loud noise (pulse). The reduction of the startle response reflects the ability of the nervous system to filter out the unnecessary information. Disruptions in prepulse inhibition response have been reported in psychiatric disorders associated with dopamine pathway dysregulation [380].

Prepulse inhibition test was conducted in four startle chambers (SR-LAB; San Diego Instruments, San Diego, CA). Each chamber consists of a clear nonrestrictive Plexiglas cylinder resting on a platform inside a ventilated box (figure 2.4). A high-frequency loudspeaker inside the chamber produces both a continuous background noise of 72 decibel (dB) and the various acoustic stimuli. Vibrations of the Plexiglas cylinder caused by the whole body startle response of the animals are transduced into analog signals by a piezoelectric unit attached to the platform. These signals are then digitized and stored in a computer.

Figure 2.4: Prepulse inhibition apparatus. Each mouse was placed into a restrainer of prepulse inhibition chamber. Underneath the restrainer, there is a piezoelectric unit that quantifies the startle response of the mouse immediately after being exposed to acoustic stimulus such as prepulses (4, 8 and 16 db above the background noise) or pulses of loud noise (120 db). The presentation, of the prepulses prior to the pulse usually diminishes the startle response. In normal (control) mice, louder prepulses cause greater degree of inhibition of the startle response.

All prepulse inhibition test sessions consisted of startle trials (PULSE ALONE), prepulse trials (PREPULSE), and no-stimulus trials (NOSTIM). The PULSE ALONE trial consisted of a 120 dB pulse and lasted for 40 msec. The PREPULSE trials consisted of a 20 msec long prepulse, an interval of 100 msec of background noise and followed by a pulse of 120 dB lasting 40 msec. Prepulse intensities were 4, 8, and 16 dB above the 72 dB background noise. The NOSTIM trial consists of background noise only. Test session started and finished with five presentations of the PULSE ALONE trial. Moreover, in between the PULSE ALONE trials, each

trial type (PULSE ALONE, PREPULSE and NOSTIM) was presented 10 times in a pseudorandom order. There was an average of 15 sec (range, 12–30 sec) break presented in between the trials (inter trial interval). The test was conducted by placing mice individually in the startle chambers, with a presentation of a 72 dB white noise as background for 10 minutes, as an acclimation period [380, 381].

The percentage of inhibition in prepulse inhibition test was calculated by using the following formula: [mean of startle response (PULSE ALONE) - mean of startle response (PREPULSE)]/ mean of startle response (PULSE ALONE) × 100.

2.3.5 Forced swimming test

The forced swimming test was used to assess depressive-like behavior. The test was performed in glass cylinders (40 cm height and 18 cm in diameter) filled with water to a level of 18 cm at temperature of 23±1°C (figure 2.5). Mice were individually placed into the cylinders and their behavior was recorder with a camera that was connected to a computer for behavioral analysis. The duration of the test was 5 minutes. During this time frame the animal activity was processed by software (FST High throughput Forced Swim Test Analysis, Biobserve, Germany www.biobserve.com). Three types of behaviors were analyzed: 1) Floating: defined as the minimal amount of movements to remain afloat, 2) Struggling: defined as energetic vertical paddling against the container wall and elevating the head above the water level, 3) Swimming: energetic movements inside the container in a horizontal- manner. After this test, mice were taken back to their cages and dried with paper towels. The total time that each mouse spent struggling, swimming or floating was used in the final analysis. This test considers that an animal displays depressive-like behavior when it tends to float more and spend less time swimming and/or struggling.

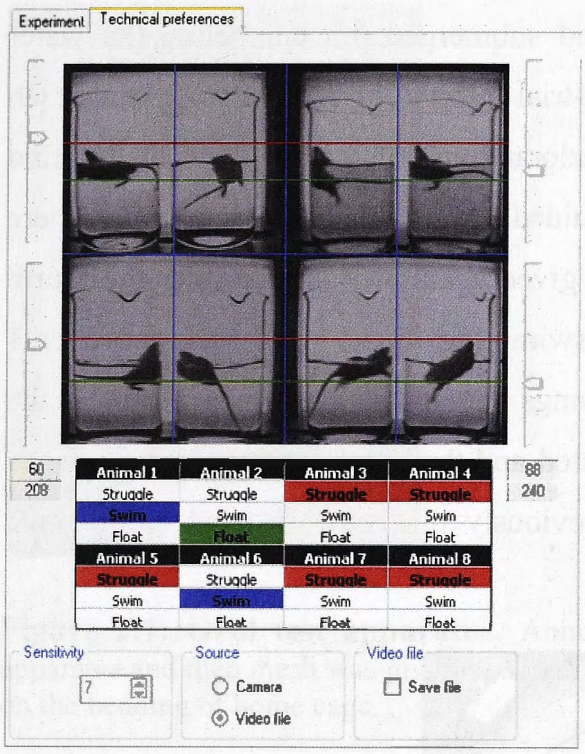


Figure 2.5: Forced swimming test. This picture depicts the different types of recorded behavior. For instance the Animal 1 is swimming, Animal 2 is floating and Animal 3 is struggling.

2.3.6 Morris water maze test

The Morris water maze is a method used to assess spatial memory in rodents. This methodology is based on the animal`s ability to memorize the position of a submerged platform. In that process rodents rely on distal cues that are placed on the rim of water tank to navigate from their starting point towards the platform. The test was performed in a water tank (1m in diameter) (figure 2.6) that was divided into 4 quadrants and designated as following: east (E), west (W), south (S) and north (N). The water inside the tank was colored (opaque white paint, by adding non-toxic water-soluble paint). The temperature was set at constant 23±1°C. Above the water tank, a camera was attached to the roof and connected to a computer operated by Biobserve software (Morris water maze, www.biobserve.com). Prior to testing, mice were placed into water to swim for 1 min without the escaping platform. On the second day, mice were given 1 min period to find a platform that was slightly elevated above the water level and with a bright flag attached to it as a cue. Mice were placed in the water facing the rim of the tank and they are given 4 trials. After each trial the platform was randomly placed in different quadrants. This initial test was used for the assessment of habitual learning [382]. During the next three days mice were exposed to the same test with few additional changes. The platform was

kept in a middle of only one quadrant and submerged 0.5 cm below the water surface; mice were given 4 trials with inter-trial interval of 5 minutes starting each time from a different side of the pool in pseudorandom order. Mice that failed to find the platform in a period of 1 minute were guided towards the platform and left there for 15 seconds. On the last day, mice were given again 4 trials, but after the fourth trial the platform was removed and their swimming tracks were recorded over 1 minute. In that last trial, the number of crossings mice made by swimming across the place where the previous platform was located and the number of their visits to the right quadrant where the platform was previously placed were used in the final analysis [382, 383].

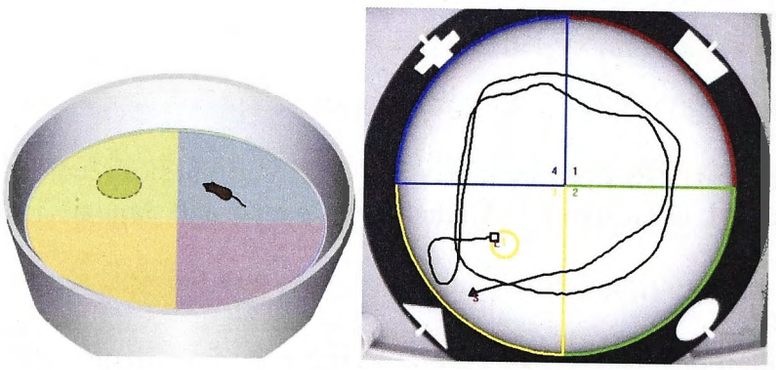


Figure 2.6: Morris water maze test. Presented pictures depict the four quadrants that were determined in a computer with the software. Mice swimming tracks to the platform were recorded as shown. Four navigational cues were attached on the rim of water tank.

2.3.7 Grid test (the four limb hang test)

The grid test is used to evaluate the strength of mouse limbs [384, 385]. In this thesis, the grid test was used as a control test to determine if the observed differences in motor coordination displayed by different mice in the rotorod test could be due to reduced muscle strength. The test aims at determining the ability of the animal to grasp the wire mesh with both forelimbs and hind limbs and to remain clinging in the inverted positions for a period of time. The latency to fall was recorded in seconds and analysed. Mice were given three trials, separated by intertribal intervals of 5 minutes. The height of the apparatus is between 20-50 cm to prevent that the animal climbs down easily (figure 2.7).

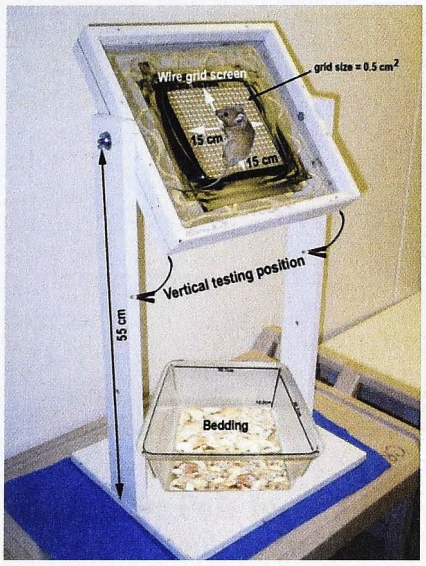


Figure 2.7: Grid test apparatus. Animal was placed on the wire mesh of the grid apparatus and then mesh was inverted. Mice were left clinging on the wire mesh till they fall on the bedding of home cage.

2.3.8 Novelty suppressed feeding test

This test is performed in order to assess anxiety/depressive-like behavior in animals [386]. It is a behavioral paradigm where a food-deprived animal has to overcome its natural fear of a novel environment and approach to food pallet in the centre of the arena in order to feed. The faster they do it, the less anxious they are. Prior to the experiment, mice were weighed and their food was removed from their cages, but the animals were not water deprived. Sixteen hours later, mice were transferred to a testing room and allowed to acclimate to the new environment by placing them in a new clean cage for a period of 30 minutes. The testing arena consisted of a plexiglass cage that was covered by bedding of 2 cm (figure 2.8). The bedding in the testing arena was changed after each tested animal and the cage cleaned in order to prevent the following animal being distracted by the odour of the previous one. In the centre of the testing arena, a small piece of previously weighted food was placed on circular filter paper (9.5 cm in diameter). Light in the testing room is dimmed and the testing arena was isolated from any noise. Each mouse was individually placed in the corner of the testing cage and time was recorded until the first moment an animal started to bite the food pellet. Afterwards each mouse was put back in its original home cage with the food pallet and left there to feed for another 5 minutes. Once this session was over, leftover food was weighed and the animal was placed back in another clean cage, supplied with water and food. At the end of the experiment, the elapsed time from initiation to feed and food weight was analysed. The measured elapsed

time represents the index on anxiety-like behavior. Longer latency to approach to the food in the centre of arena represents anxiety-like behavior. The measurement of food intake in the home cage was used as a control test to verify the anxious behavior that mice might have potentially displayed in new environment. The home cage is a familiar environment and therefore this second part of the experiment is used to determine mouse food intake in a non-stressful environment.

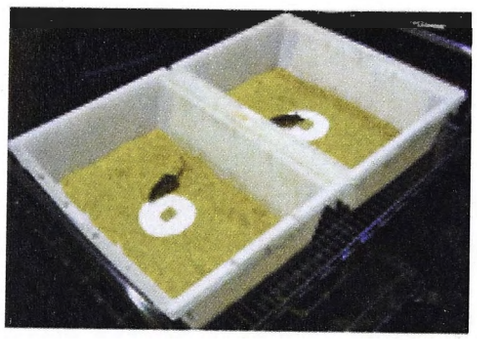


Figure 2.8: Novelty suppressed feeding test arena. The picture depicts the arena where mice are tested. In the centre of the arena, food pallet is placed on the circular filter paper. Latency for each animal to start feeding is measured in seconds.

2.3.9 Pole test

This test represents one of the possible methods used to assess the agility of the animals. It is mostly used as a measurement of bradykinesia (slowness of movement) [387], a locomotor disability most frequently seen in Parkinson`s diseased patients. The test requires the usage of pole, 50 to 55 cm in height and 8 to 10 mm in diameter with rough surface that stands vertically in a home cage (figure 2.9). The test was conducted by placing an animal close to the top of the pole and leaving the animal`s head up. The period of time that animal spent in turning around (the first measurement) and climbing down (the second measurement) the pole was recorded. This task is very sensitive to nigrostriatal dysfunction as it involves skilled forelimb grasping and maneuvering, which would require an intact basal ganglia [388]. Therefore, both parameters (time to turn around and climb down) were used for assessment of bradykinesia in mice.

Figure 2.9: Pole test. Mice are individually placed on the top of the pole and time to turn around and climb down the pole are recorded in seconds.

2.3.10 Stepping test

The stepping test is another reliable method to assess forelimb akinesia caused by bilateral dopaminergic neuron lesion in a parkinsonian mouse model [389]. It is performed on a table and the animal is placed on one edge of it. Afterwards, hind legs are lifted by pulling animal`s tail upwards and in that position the animal is pulled back, toward the other edge of the table. The number of animal`s adjusting steps from both forepaws are counted (figure 2.10) [389].

Figure 2.10: Stepping test. The picture depicts the method used in stepping test. Mice hind limbs are lifted by pulling animal`s tail upwards and thereafter mice are pulled back towards the other edge of the table. Number of adjusted steps are recorded.

2.3.11 Hind limb clasping test

This test is used for an assessment of dyskinesia (diminished voluntary movements) in mice [390]. Mice are suspended in the air by the tail for a period of ten seconds (figure 2.11). The position of hind limbs are observed and scored. Based on the position of the limbs mice are scored as:

0- hind limbs are splayed outwards,

1- one hind limb is retracted towards abdomen,

2- both hind limbs are partially retracted towards abdomen and

3- hind limbs are completely retracted and touching the abdomen [391].

Figure 2.11: Hind limb clasping test. The picture depicts the three examples of mice hind limb positions. Mouse on the very left side is scored 0; mouse in the middle is scored 2 and mouse on the right hand side is scored 3.

2.4 L-DOPA/carbidopa test

L-DOPA/carbidopa was prepared in concentration of 30mg/ 3mg/kg [376]. L-3,4-dihydroxyphenylalanine (L-DOPA) was purchased from Sigma-Aldrich and was dissolved in distilled water for 20 minutes before injection. Carbidopa (Sigma-Aldrich) was mixed in a drop of Tween-20 and distilled water, and added to the already prepared L-DOPA solution. The mixture of L-DOPA/carbidopa was briefly sonicated in order to completely dissolve these mentioned drugs.

Twelve months after the first saline/LPS injection, WT mice were subjected to L-DOPA/carbidopa test. Prior to testing, mice were pre-trained for four days in order to establish stable baseline performance on the rotarod apparatus. On the day of the experiment, mice were tested on the rotarod apparatus and the results were recorded as initial baseline performance. Afterwards, mice were injected intraperitoneally with the L-DOPA/carbidopa solution and their locomotor activity was tested on a rotarod apparatus after two hours. In order to show that potential positive effect of L-DOPA/carbidopa injection on locomotor activity was only due to replacement of missing dopamine, mice were subjected to another rotarod test a week later. It is considered that complete effect of L-DOPA/carbidopa wears out during that period of time [376].

2.5 Metabolic studies

2.5.1 Dexa scan

Dual-emission X-ray absorptiometry (DEXA) scan is used primarily to evaluate bone mineral density (BMD), body composition and fat content [392]. Prior to testing, animals are deeply anesthetized with 2-3% isofluorane-oxygen gas for an

entire period of test (5-10 minutes) and then placed in a PIXImus apparatus (PIXImus Mouse Densitometer). The whole body of each mouse was scanned and analyzed with PIXImus software (LUNAR PIXImus 2) (figure 2.12). The mouse head was excluded from the scan by using the ROI (region of interest) that was placed manually surrounding the whole body. The data that were used for further analysis were: percentage of fat mass, weight of lean tissue, BMD and bone mineral content (BMC).

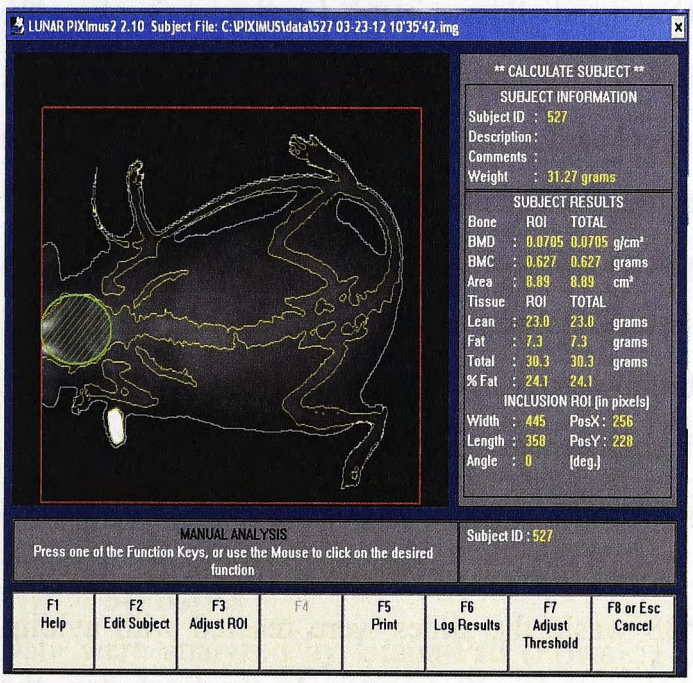


Figure 2.12: PIXImus software. The picture depicts the scan of mouse whole body and parameters such as: BMD, BMC, percentage of fat mass and weight of lean tissue.

2.5.2 Intraperitoneal glucose tolerance test (IPGTT)

The IPGTT test is used to assess how fast the glucose is cleared out from the blood upon glucose (i.p.) injection. Prior to testing, mice were transferred to clean cages and fasted for a period of 6 hours, but they were provided with water ad libitum. Mice were weighed and their baseline blood glucose levels were checked by collecting one small drop of blood from animal`s tail vein at 0 minute (baseline). Afterwards, mice were injected with D-glucose (2 g/kg, i.p.) solution [393], which was previously dissolved in phosphate buffered saline (PBS). The glucose levels were measured by a glucometer (Accu Chek®, Roche, Australia) at 30, 60, 90 and 120 minutes after the injection.

2.6 Immunoassays

2.6.1 Immunohistochemistry of dopamine neurons and microglia cells

Mice were anesthetized with intraperitoneal injection of ketamine/xylasine (100+10 mg/kg, ratio 2:1) and transcardially perfused with solution of cold PBS and heparine (5 U/ml) within 3 minutes. The mouse brain was removed and frozen in chilled isopentanol and stored at -80°C. The brain blocks were dissected in a cryostat (set at -12°C) in 15 µm thick coronal sections that encompassed the entire substantia nigra. The sections were collected on gelatin-coated slides, air-dried and post-fixed in 4% paraformaldehyde (pH~7.4 in PBS) for 8 to10 minutes. After a PBS wash (3 times within 5 minutes), the sections were incubated for 20 minutes in 3% peroxide mixed in methanol in order to inactivate the endogenous peroxidase. Afterwards, the sections were incubated with 5% normal goat serum and 0.015% Triton X-100 in PBS. After 30 minutes incubation, the sections were immunoreacted with rabbit anti-tyrosine hydroxylase antibody (1:1000 dilution, Life Technology) and rat anti-mouse CD68 monoclonal antibody (1:700, AbD Serotec) in 0.015% Triton X-100 for 24 hours at 4°C. The next day, the slides were washed in PBS and incubated with biotinylated goat anti-rabbit or goat anti-rat secondary antibody (Vector Laboratories) for 1 hour. After a PBS wash, the slides were reacted with avidin-biotin complex (ABC, Vector Laboratories) for 30 minutes. In the final step, the slides were washed with PBS and incubated with 3,3`-diaminobenzidine (DAB, Sigma) and 30% peroxide until the color was developed (5-7 minutes). The slides were counterstained with hematoxylin (30 seconds), dehydrated in alcohol gradient (80%, 90%, 100%, 100%), immersed in xylene, mounted with DPX and finally coverslipped.

2.6.1.1 Quantification of dopamine neurons and microglia

In order to assess the loss of dopamine neurons, thirty-two consecutive slides were collected in duplicates. Thickness of slides was 15 µm and the region of substantia nigra (rostral to caudal: -2.65 to -3.61 mm posterior from bregma) was sampled. Eight evenly spaced slides were used for counting the dopamine neurons or microglia. The borders of substantia nigra were defined in order to exclude the VTA area from counting [394]. The area defined for counting covered an entire area from rostral part of SNpc to the caudal end of SNpr. The images of the slides were taken on an Olympus camera (20 x magnification) and analyzed by ImageJ software (from

NIH). Positive CD68 microglia cells in substantia nigra were counted manually on a Nikon Eclipse 50i microscope (40 x magnification).

2.6.2 The enzyme-linked immunosorbent assay (ELISA)

Blood was collected transcardialy in ethylenediaminetetraacetic acid (EDTA)-coated tubes. Blood samples were centrifuged at 2000g for 15 minutes at 4°C and plasma was separated from blood cloth and stored at -20°C until used. The samples for insulin measurement were diluted in ratio of 1/8 for HFD-fed mice, 1/4 for Casp-1-/- mice, while samples of RD and IL-1ra-/- were not diluted. Plasma insulin was measured with ALPCO ELISA kit (Mouse Ultrasensitive Insulin ELISA, catalog number: 80-INSMSU-E01, E10). Leptin levels were measured with mouse Leptin ELISA kit (DuoSet R&D Systems, catalog number: DY498). Samples for leptin measurements were diluted in ratio 1/3 for Casp-1-/- and RD-fed mice and 1/8 for HFD-fed mice. MCP-1 plasma level analyzed with Mouse/Rat CCL2/JE/MCP-1 ELISA kit (Quantikine ELISA, catalog number: MJE00, SMJE00, PMJE00), according to the manufacturer`s instructions. Samples for MCP-1 measurements were diluted in ratio of 1/2 for RD and HFD-fed mice, Casp-1-/- and IL-1ra-/-.

2.7 Statistics

Data were analyzed by Graph-Pad (Prism 5) software and expressed as means ± S.E.M. Time-dependent differences (between baseline and final performance) in behavioral and metabolic outcomes were analyzed within each treated group (WT, Casp-1-/- and IL-1ra-/-) by paired Student`s T-test. Unpaired Student`s T-test was used to analyze the differences between RD and HFD-fed mice. One-way ANOVA followed by Tukey`s post-hoc test was used to analyze the differences among the three genotypes (WT, Casp-1-/- and IL-1ra-/-). Values of $p<0.05$ were considered significant.

3. Effect of peripheral inflammatory challenge and high-fat diet on behavioral outcomes.

3.1 Introduction

As it was previously described in the general introduction (chapter 1.2), LPS-induced activation of the innate immune system increases inflammatory factors such as IL-1β and TNF-α that are able to propagate the inflammatory cascade within the CNS by activated microglial cells. Proinflammatory factors produced by local microglial cells might interfere with normal synthesis of dopamine, its secretion and reuptake (chapter 1.10.5.1). Moreover, IL-1 has been directly implicated in programmed cell death of dopamine neurons (chapter 1.10.3). Furthermore, emerging evidence suggests that high-fat diet-induced obesity causes low-grade chronic inflammation that, in concert with metabolic disorders such as leptin and insulin resistance, may further dysregulate the function of the dopamine system (chapter 1.11). Thus, dysregulation of the innate and adaptive immune system could lead to the alteration of locomotor skills.

Impairment of the dopamine system may be effectively assessed by performing behavioral paradigms, such as open-field and rotarod tests. Evaluation of neurological/motor capabilities in rodents, through the implementation of various behavioral tests, is a helpful tool to define the phenotype of mice with neurological impairment due to the dysfunction of the basal ganglia. The open-field and rotarod test offer the opportunity to examine the extent of locomotor disabilities caused by the alteration of the dopaminergic system, which is usually manifested by reduced locomotion, impaired coordination and postural instability, occurrence of tremors, rigidity and bradykinesia (slowness of movement). Other behavioral tests such as the stepping test and the pole test also represent noninvasive methods for the assessment of motor skills [395].

Dysregulation of the nigrostriatal system may also be accompanied by the occurrence of other, non-motor signs of behavioral impairment, such as anxiety, depression [396] and cognitive decline [397]. The reported prevalence of cognitive decline in the form of dementia in Parkinson`s disease patients was estimated around

31% [398] and the prevalence of depression was estimated to occur in 35% of Parkinson`s diseased patients [399, 400]. Evaluation of anxiety-like behavior in rodents can be ascertained with tests such as the elevated plus maze, novelty suppressed feeding and open-field. Depressive-like behavior can be assessed by the forced swimming test. Cognitive decline in mice may be measured by tests such as the Morris water maze, which is designed for an assessment of spatial learning and memory, and the prepulse inhibition test, which is used for the evaluation of animal attention once exposed to silent stimuli from the environment [401].

3.1.1 Evaluation of neurological/motor skills

General control over locomotor activity is highly dependent on normal functionality of basal ganglia and sufficient production of dopamine by its neurons. Dysfunction in basal ganglia in Parkinson`s diseased patients results in impairment to initiate voluntary movements, maintenance of gait posture and inability to suppress involuntary movements [402].

The inflammatory process may dysregulate the normal function of the dopamine system via proinflammatory cytokines that originate from peripheral organs and/or brain resident immune cells. For instance, a single intraperitoneal injection of LPS into male WT mice (5 mg/kg) was sufficient to cause 23% depletion of dopamine neurons in substantia nigra over a period of seven months [109, 376]. In the same study, loss of dopamine neurons (37%) after nine months in five monthly LPS-injected female mice was accompanied with decreased locomotor skills, as it was ascertained in the rotarod apparatus and in the open-field arena [376]. Histological analysis of the brains showed pronounced activation of microglia and increased immunostaining of IL-6 [376]. In another similar mouse study, as the serum level of TNF-α declined to baseline, examination of the brain extracts showed that protein levels of TNF-α [109] and IL-18 [403] were still elevated even ten months after the first LPS (5 mg/kg, i.p.) injection. Collectively, these studies show that a long-term activation of the CNS inflammatory cascade after a peripheral LPS inflammatory challenge [109, 376, 403] was associated with nigrostriatal dopamine degeneration and locomotor disabilities [376]. Human study has shown that frequent exposures to pesticides, which is mostly observed in agricultural communities makes people more susceptible to develop Parkinson`s disease [404]. Pesticides are known for their ability to induce brain inflammation and have been related to occurrence of

Parkinson`s disease [405]. Therefore, inflammation induced by endotoxins may cause deleterious effects on the survival of dopamine neurons, which ultimately will result in impaired locomotor skills.

Even though the exact cause of dopamine cell loss in Parkinson`s disease is unknown, factors such as genetics, age, diet, exposure to environmental pollutants and various toxins are thought to be implicated in the development of this disease [406]. Since there are many factors that may contribute to the onset or progression of Parkinson`s disease, it was proposed that these factors, or "hits" may "prime" the neuroiflammatory cascade in the brain in additive or synergistic manner [407]. According to this hypothesis of "multiple hits", obesity might be considered as one of the factors that along with inflammation may lead to dopamine neuronal degeneration [406, 408]. Obesity and high-fat diet-induced insulin resistance may favor an increase of the susceptibility to dopaminergic neurodegeneration [408]. High-fat diet-induced insulin resistance may interfere with normal release and reuptake of dopamine into presynaptic vesicles [12]. Data from previous study suggest that rodents fed a high-fat diet have decreased availability of D2 receptors in striatum accompanied with increased levels of ROS [367]. Feeding mice a high-fat diet has been shown to exacerbate the depletion of striatal dopamine under treatment with the neurotoxin MPTP [409] followed by the depletion of dopaminergic terminals [351]. The insulin resistance in high-fat diet-fed rats was suggested to lower the threshold for the depletion of nigrostriatal dopamine content with unilateral infusion of 6-OHDA in medial forebrain bundle [353]. Overall, these studies suggest that high-fat diet consumption and obesity exacerbated the depletion of nigrostriatal dopamine [351, 353, 409]. Thus, a high-fat diet may elicit metabolic and inflammatory dysregulations leading to dopaminergic neurodegeneration.

In section 3.4.1 the putative role of the IL-1 pathway was assessed on motor ability in mice of different genotypes (WT, Casp-1-/- and IL-1ra-/-) that were injected with saline and/or LPS (single or five monthly injections). In the same section, the effect of long-term exposure to HFD on locomotor skills of WT mice was ascertained.

3.1.2 Evaluation of an anxiety/depressive-like behavior

Chronic activation of the immune system has also been related to depressive behavior in animals and humans, since it is well known that animals injected with

LPS develop sickness behavior [410] and depressed patients frequently have increased serum concentration of proinflammatory factors [411-415]. In particular, an increasing body of evidence suggests that patients suffering from rheumatoid arthritis, an example of chronic inflammatory disorder, are twice as likely to suffer from depression [416, 417]. Therefore, it has been thought that immune challenges and secretion of cytokines may play a role in the development of depression. For example, several cytokines have been found to be elevated in the plasma of depressed patients, such as IL-1 [412], IL-6 [413], IFN-γ [414] and TNF-α [415].

Emerging new evidence suggests that dopamine neurons from the VTA zone modulate a neuronal circuit that is involved with the control of depressive-like behavior in rodents [418]. It was demonstrated that selective inhibition of dopamine neurons in the VTA zone induces depressive-like of behavior in mice [418] suggesting that neuroinflammation may interfere with dopaminergic neurotransmission and consequently cause depressive-like of behavior in rodents.

Endotoxin-induced inflammation may regulate depressive-like behavior in rodents. Symptoms seen in sickness behavior induced by the acute administration of LPS (hypomotility, hypophagia, decreased exploratory activity, anhedonia) are mostly observed in depression. One of the elements that reflect depressive behavior is anhedonia (inability to experience pleasure). As such, it has been recently reported that LPS induces the release of cytokines in the brain that in turn activate monoamine transporters to metabolize monoamines leading to anhedonia [419]. Likewise, the same research team reported that a LPS-induced anhedonia over-activates serotonin transporters and consequently increases serotonin reuptake [420]. This relation between LPS and serotonin receptors is connected through LPS activation of TLR4, which stimulates the activation of the p38 MAPK pathway [421] that results in activation of serotonin reuptake [422].

As it was previously described in chapter 1.11, obesity causes a low-grade inflammatory state [6]. Human studies have shown that obesity increases the risk of depression [423]. In regard to the concept of low-grade chronic inflammation caused by obesity, it has been shown that high-fat diet consumption for ten weeks exacerbated the depressive-like behavior in the Flinders Sensitive and Resistant Line (FSL/FRL) rats, a genetic rat model of depression [424].

Depressive-like behavior in rodents may be evaluated with the behavioral paradigm such as forced swimming test [425]. As it was explained in the methods section, this test evaluates the latency to "give up" as measure of depressive-like behavior. For example, VMAT-2 heterozygous mutant mice displayed depressive-like behavior, measured as increased floating time in the forced swimming test [426]. Therefore, the effect of single and/or repeated LPS injection in absence or presence of a high-fat diet on depressive-like behavior will be shown in chapter 3.4.2.

There is evidence suggesting that anxiety-like behavior in rodents may be influenced by neuroinflammatory process in the brain [427]. High levels of IL-6 and TNF-α expression has been reported to be associated with anxiety in a mouse model of autoimmune lupus [428]. Likewise, animals exposed to polymicrobial sepsis showed signs of anxiety in elevated plus maze without locomotor impairment and had increased levels of inflammatory markers in the brain (IL-1β, IL-6, TNF-α, IFN-γ) [429]. Similarly, inflammatory markers have been correlated with anxiety behavior in humans [430]. Deficiency of IL-1R1 in mice had a positive effect on decrease of anxiety without altering locomotor activity, suggesting that IL-1 plays a role as an anxiogenic factor [431]. Anxiety behavior in rodents is regulated via the CRH signaling pathway and its receptors CRHR1 are expressed in the anterior pituitary gland. It was recently suggested that the dopamine system could have a role in emotional disorders [432]. In particular it was proposed that CRH/CRHR1 mediate the control over glutaminergic and dopaminergic neurons in an antagonistic manner, modulating anxiety behavior [433]. In conjunction with the previous statement, it was demonstrated that activation of glutamate releasing neurons in the limbic system by CRH elicits anxiety behavior, whereas release of dopamine in mid-brain induced by CRH evokes a behavioral outcome that reduces fear [433].

Anxiety behavior in rodents is evaluated with several methods, such as the open-field test, the elevated plus maze [379] and the novelty suppressed feeding [434]. The effect of single and/or repeated LPS challenges and the possible effects of high-fat diet on anxiety behavior in mice will be described in chapter 3.4.2.

3.1.3 Evaluation of memory and cognition

The relevance of hippocampal function in learning and memory is very well demonstrated [435, 436]. Learning is a process that involves formation of new

synaptic connections between neurons that was hypothesized by Ramon y Cajal in 1893 [437]. Therefore, synaptic plasticity has been explained as a connection between two neurons being active at the same time in a process determined as long-term potentiation (LTP) followed by the dysregulation of this synaptic activity- long-term depression (LTD). Synaptic signal trafficking through LTP or LTD is enabled through α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) [438]. The amplitude of LTP or LTD induced synaptic firing is further modified by the number of NMDA receptors in a process of synaptic scaling [439]. Physiological levels of IL-1 are shown to be important for the maintenance of LTP [129] and also in the expression of AMPA receptors [440]. Similarly, impaired IL-1 signaling in IL-1R-/- mice was associated with a deficit in the hippocampal memory [441]. On the other hand, pathological conditions accompanied with increased levels of proinflammatory cytokines within the brain resulted in learning and memory impairment [442]. For instance, an excess of IL-1 was shown to induce memory impairment in mice by affecting cholinergic neurotransmission [443], nerve growth factor (NGF) [444] and impaired BDNF expression in the hippocampus [445].

High-fat diet consumption has been shown to impair memory and cognition [446]. As obesity is associated with elevated proinflammatory factors, it has been observed that cytokines might cause memory impairment [447]. Comparative studies in humans and rodents suggest that high-fat diet-induced insulin resistance is associated with the development of memory deficit and dementia [448]. It has been demonstrated that four weeks exposure to a high-fat diet impaired hippocampal neurogenesis in male rats before causing obesity, which was associated with high serum levels of corticosterone [449]. These data collectively suggest that chronic high-fat diet consumption and obesity-induced metabolic perturbations may lead to memory impairment [446-448].

In rodents, impaired dopaminergic neurotransmission in the prefrontal cortex was reported to impair working memory function [450]. Stimulation of dopamine release in the prefrontal cortex of Parkinson`s diseased patients with antidepressants [451] improved the memory deficit [452]. In regards to the effect of diet-induced obesity, it has been demonstrated that electrically evoked dopamine release in prefrontal cortex of obesity-prone rats was attenuated [453], which might suggest possible link

between obesity-induced dysregulation of the dopamine system and memory impairment.

Memory deficit in rodents can be evaluated by several behavioral paradigms such as the Morris water maze [454] and the prepulse inhibition test [455]. The role of IL-1 signaling and high-fat diet consumption on memory impairment will be addressed in chapter 3.4.3.

3.2 Objectives

Single and/or repeated LPS inflammatory challenges and high-fat diet consumption may potentially interfere with normal functionality of the basal ganglia. Therefore, the aims are to ascertain the putative role of the IL-1 pathway and high-fat diet-induced obesity on:

1) Locomotor skills;
2) Anxiety/depressive-like behavior and;
3) Memory and cognitive function in mice.

3.3 Brief methodology

Different mouse genotypes such as WT, Casp-1-/-, and IL-1ra-/- were tested in batteries of behavioral tests in order to assess:

1) Locomotor capabilities: Possible alteration in motor abilities in mice was tested twice a month in the open-field and rotarod paradigms. These two tests were performed over a period of nine months starting from the first saline/LPS injection. This time frame was chosen as it was previously shown that repeated systemic LPS injections in female mice were sufficient to induce motor disabilities in the rotarod and open-field arena in a period of seven to nine months, respectively [376]. After nine months, another set of tests was employed to assess locomotor functions in mice: The grid test (was used as a control test for assessment of muscle strength in high-fat diet-fed mice), the pole test (to assess bradykinesia), the stepping test (to assess akinesia) and the hind limb clasping test (to assess dyskinesia).
2) Anxiety/depressive-like behavior in mice was assessed with behavioral tests such as the open-field (central/total distance), the elevated plus maze, the novelty suppressed feeding and the forced swimming test. Except for the open-field test, the other tests were performed between 10 and 13 months

after the first saline/LPS injection. Tests were organized and performed in an orderly manner by exposing the mice from the least to the most stressful test.

3) Memory and cognitive function: Mice were tested after a period of 10 to 13 months starting from the first saline/LPS injection in the Morris water maze for an assessment of memory and in the prepulse inhibition test for cognitive evaluation.

3.4 Results

3.4.1 Evaluation of neurological/motor skills

In order to better understand the long-term effects elicited by single or repeated LPS inflammatory challenges and in particular the role of IL-1 on dysregulation of the nigrostriatal system, several behavioral tests were performed to assess locomotor skills in mice.

The open-field test is used as one of the methods for an assessment of voluntary movements in rodents. In this test total traveled distance is recorded and used as a measure of mouse general motor activity. For instance, a decrease in dopamine level, as it was shown on the example of VMAT-2 deficient mice, was accompanied by a reduction in locomotor activity in the open-field test that was later rescued with L-DOPA treatment [252]. As it was previously described, a loss of dopamine neurons (37%) led to significant decline in traveled total distance [376]. Therefore, the open-field test has been used as a reliable indicator of the loss of dopamine neurons and/or level of dopamine [376].

Figure 3.1 (A, C and E) displays the total distance travelled (measured in cms) in the open-field arena of mice of the three studied genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively). Data collected at the baseline point (0 month) and after 9 months from the first saline/LPS injection are presented for the three experimental conditions (Sal, LPS-1T and LPS-5T). The main purpose of these three figures is to evaluate the putative time-dependent differences in locomotor activity between time 9 months and 0 month for each treatment within each genotype.

Figures 3.1 B, D and F were constructed to evaluate the possible differences among the three experimental conditions for each genotype (WT, Casp-1-/- and IL-1ra-/-, respectively). The baseline point (0 months) is considered in each experimental condition to be 100%. The absolute difference (travelled distance at 9 months –

travelled distance at 0 month) was expressed as a percentage of change from time 0 month for each experimental condition.

The total distance travelled in the open-field arena did not change significantly within the WT or Casp-1-/- groups neither within the treatment group (difference between 0 and 9 month) (figure 3.1 A and C, respectively) nor among treated groups (Sal, LPS-1T and LPS-5T) (figure 3.1 B and D).

With regards to the IL-1ra-/- mice, the total travelled distance largely decreased at 9 months for each experimental condition (Sal, LPS-1T and LPS 5-T) (figure 3.1 E). The percentages of decreases versus their time 0 month were 53% for saline ($p<0.001$), 31% for LPS-1T ($p<0.01$) and 37% for LPS-5T ($p<0.01$) (figure 3.1 F). The comparison among experimental groups yielded no significant differences (figure 3.1 F).

Figure 3.1 G and H were constructed to evaluate the genotype differences for each experimental group at baseline (time 0 month) (figure 3.1 G) and after 9 months (by considering the percentage of the difference between 0 and 9 months calculated in figures 3.1 B, D and F and sorting the data per experimental condition (figure 3.1 H)).

As it is shown in figure 3.1 G there were significant differences among the three genotypes at the baseline level. Casp-1-/- mice were 39% more active than WT mice ($p<0.001$) and 58% more active in comparison to IL-1ra-/- mice ($p<0.001$). In regard to the differences in motor activity that was already shown with Casp-1-/- mice, IL-1ra-/- mice were also 32% less active in comparison to WT mice ($p<0.001$).

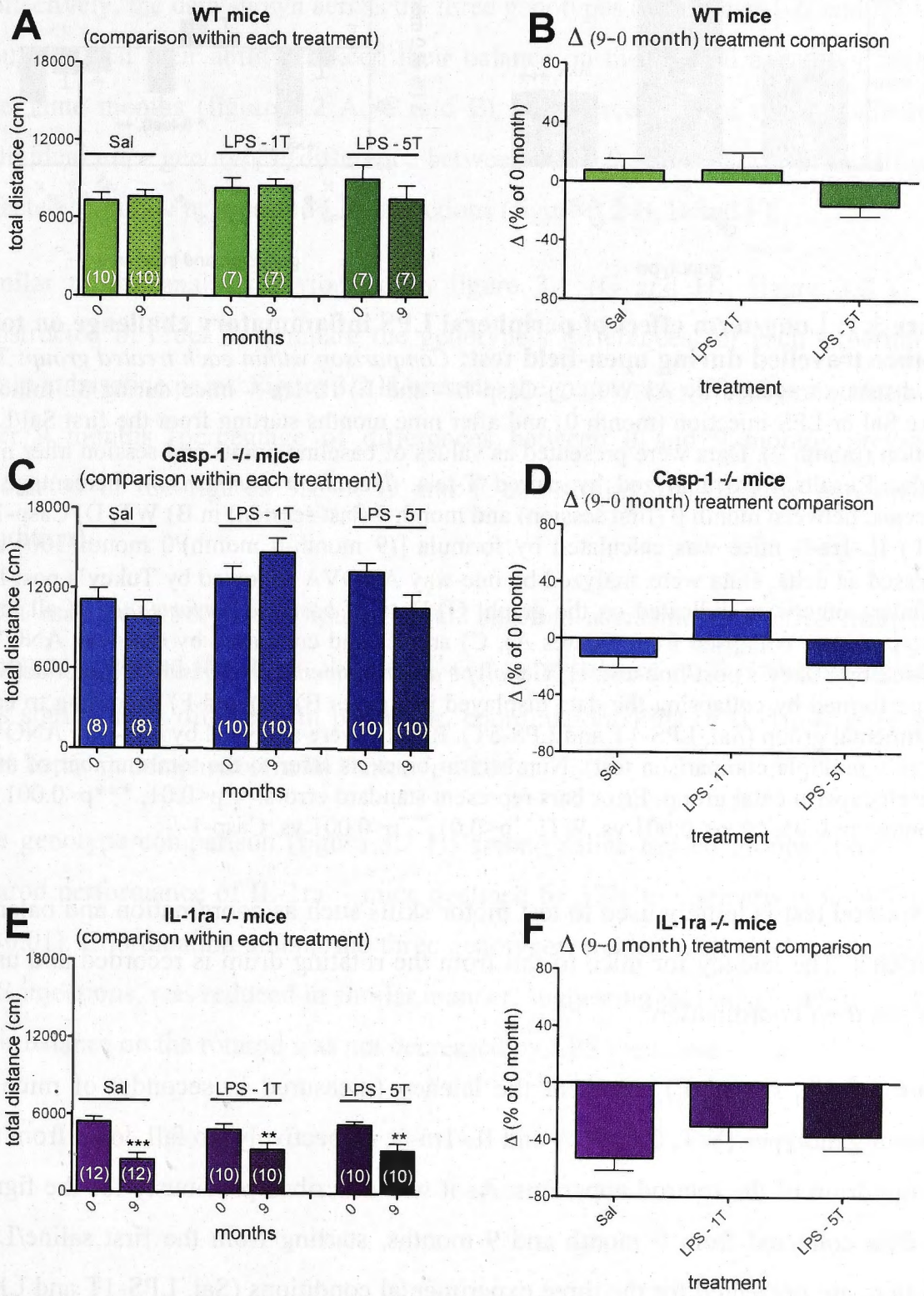
As it was already described in the figure 3.1 G, there were significant differences in the locomotor activity at the baseline among the three genotypes. In order to understand if the final reported traveled distance in the open-field test is different among three different strains of mice, percentages of changes that were previously calculated for the individual genotype (figure 3.1 B, D and F) were gathered in figure 3.1 H and compared among genotypes within same treated group.

In figure 3.1 H, among saline treated groups, locomotor activity of IL-1ra-/- mice was significantly reduced by 60% in comparison to WT ($p<0.001$) mice and 40% versus Casp-1-/- mice ($p<0.01$). The similar situation was observed in a group

treated with one LPS injection, while this trend did not persist in a group injected five times with LPS.

Overall, presented data from the open-field test suggest that there were not obvious LPS effects observed among the three genotypes. However, the locomotor activity of IL-1ra-/- mice significantly reduced during nine months period, suggesting that the absence of IL-1ra could have impaired nigrostriatal activity.

Open-field test



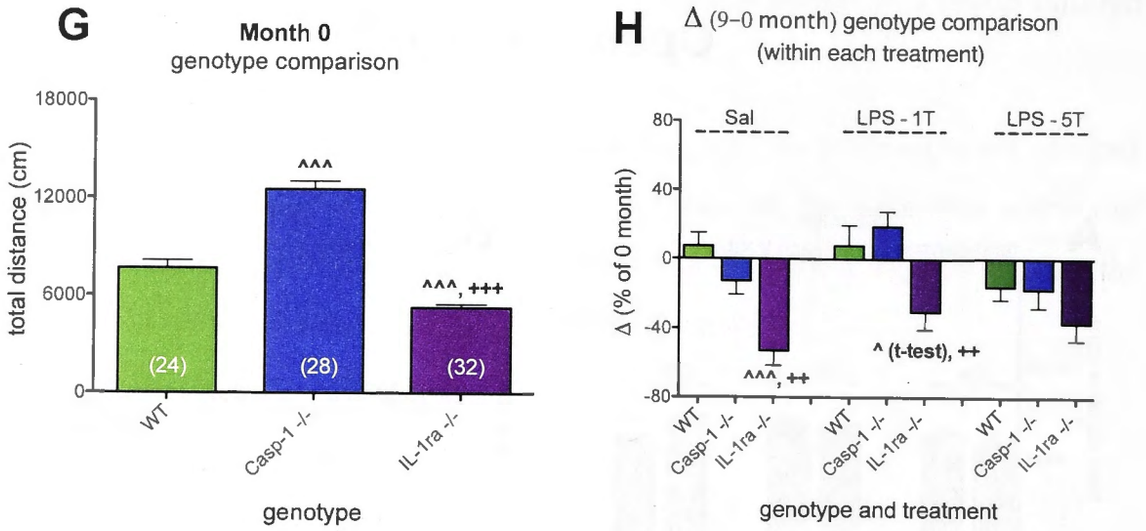


Figure 3.1: Long-term effect of peripheral LPS inflammatory challenge on total distance travelled during open-field test: *Comparison within each treated group*: The total distance travelled by A) WT, C) Casp-1-/- and E) IL-1ra-/- mice during 32 minutes before Sal or LPS injection (month 0) and after nine months starting from the first Sal/LPS injection (month 9). Data were presented as values of baseline points and session after nine months. Results were analyzed by paired T-test. *Treatment comparison*: Percentage of difference between month 0 (first session) and month 9 (last session) in B) WT, D) Casp-1-/- and F) IL-1ra-/- mice was calculated by formula [(9 month-0 month)/0 month*100] and expressed as delta. Data were analyzed by one-way ANOVA followed by Tukey`s post-hoc test unless otherwise indicated on the graph. G) Data of *baseline performance* of all three genotypes were collapsed from figures A), C) and E) and compared by one-way ANOVA followed by Tukey`s post-hoc test. H) *Genotype and treatment comparison* of motor activity was performed by collapsing the data displayed in figures B), D) and F) according to each experimental group (Sal, LPS-1T and LPS-5T). Results were analyzed by one-way ANOVA (Tukey`s multiple comparison test). Numbers in brackets refer to the total number of mice per each experimental group. Error bars represent standard errors. **p<0.01, ***p<0.001 vs. 0 month; ^p<0.05, ^^^p< 0.001 vs. WT; $^{++}$p<0.01,$^{+++}$p<0.001 vs. Casp-1-/-.

The rotarod test is largely used to test motor skills such as coordination and balance in rodents. The latency for mice to fall from the rotating drum is recorded and used to assess their coordination.

Figure 3.2 (A, C and E) represent the latency (measured in seconds) of mice of different genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) to fall down from the rotating drum of the rotarod apparatus. As it was described previously for the figure 3.1, data collected from 0 month and 9 months, starting from the first saline/LPS injection, are presented for the three experimental conditions (Sal, LPS-1T and LPS-5T) in order to assess changes in animal`s coordination in a time-dependent manner.

Figures 3.2 B, D and F were constructed to evaluate the effect of single and repeated LPS injections on animal coordination within each genotype (WT, Casp-1-/- and IL-

1ra-/-, respectively). As it was described in figure 3.1 (B, D and F), baseline points collected at 0 month are considered for each experimental condition as 100% and percentage of difference for each treatment was calculated.

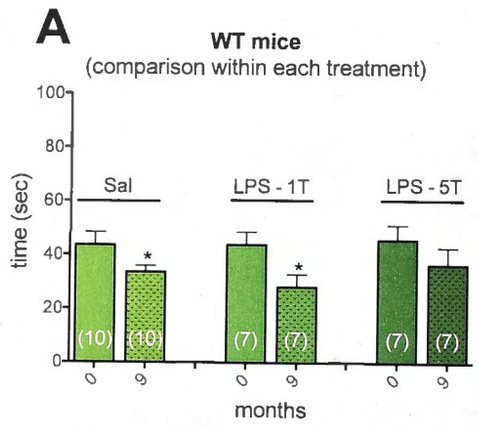
Collectively, the data shown across the three genotypes (WT, Casp-1-/- and IL-1ra-/-) suggest that their ability to keep their balance on the rotarod apparatus declined after nine months (figure 3.2 A, C and E). The percentage of decline within the individual mice genotypes (difference between 0 and 9 month) was not significantly affected by single or repeated LPS injections (figure 3.2 B, D and F).

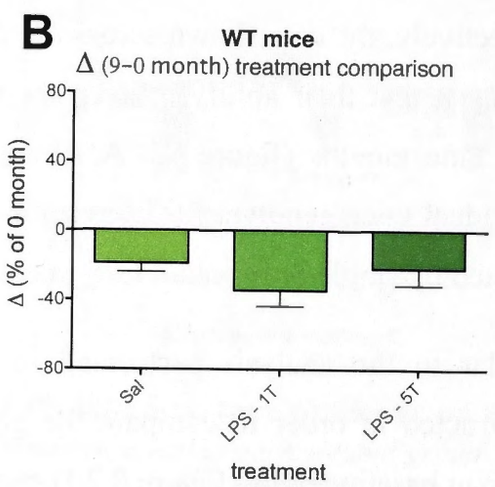
Similar to the analysis performed in figure 3.1 (G and H), figure 3.2 G was constructed in order to compare the genotype`s differences for each experimental group at baseline point. Figure 3.2 H presents the genotypes difference in rotarod test after 9 months (percentage of differences between 0 and 9 months previously calculated in the figures 3.2 B, D and F and sorting the data by experimental condition).

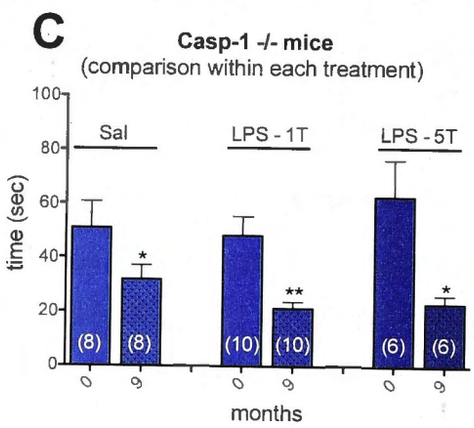
As it may be observed in figure 3.2 G, baseline assessment of mice from three genotypes showed that their ability to keep their balance on the rotarod apparatus was significantly different. In particular, coordination skills of IL-1ra-/- mice were 20% better in comparison to WT mice ($p<0.05$).

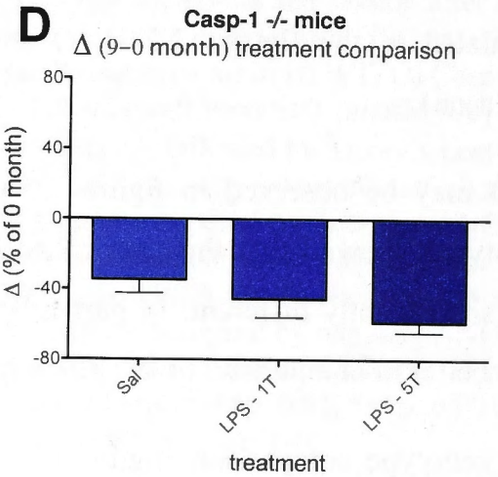
The genotype comparison (figure 3.2 H) among saline-treated groups showed that rotarod performance of IL-1ra-/- mice declined by 37% in comparison to WT mice ($p<0.01$). Coordination among the three genotypes, treated with single and repeated LPS injections, was reduced in similar manner, suggesting that mice`s ability to keep their balance on the rotarod was not decreased by LPS treatment.

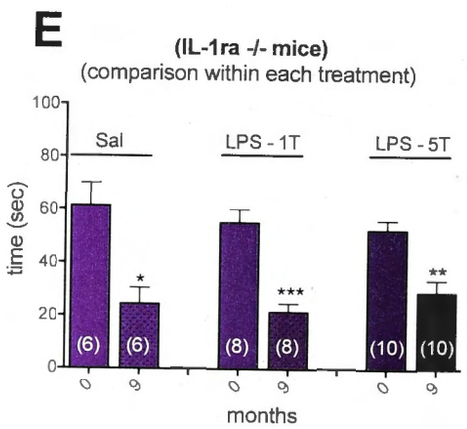
Rotarod test

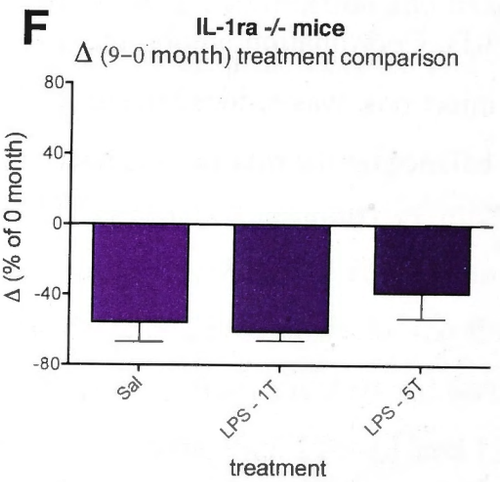












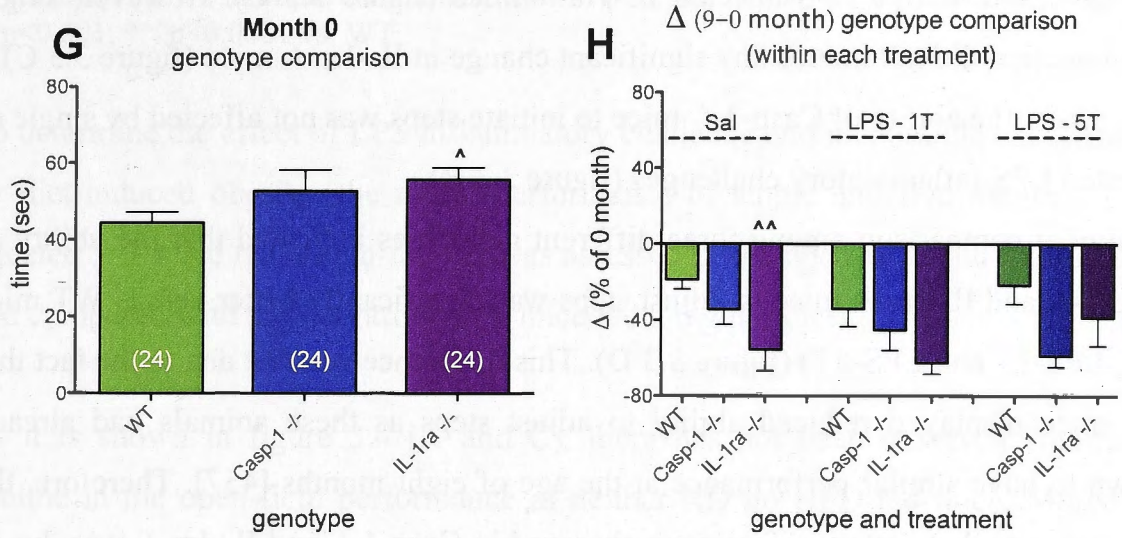


Figure 3.2: Long-term effect of peripheral LPS inflammatory challenge on rotarod performance: *Comparison within each treated group*: Average latency (measured in seconds) for A) WT, C) Casp-1-/- and E) IL-1ra-/- mice to fall down from the rotating drum. Data collected at 0 month and after 9 months were compared within each treated group by paired T-test. *Treatment comparison*: Percentage of difference between month 0 (first session) and month 9 (last session) in B) WT, D) Casp-1-/- and F) IL-1ra-/- mice was calculated for each experimental group according to the previously described formula (in the legend to figure 3.1) and analyzed by one-way ANOVA followed by Tukey`s post-hoc test. G) Data of *baseline performance* in rotarod test of three genotypes were gathered from figures A), C) and E) and differences among genotypes were compared by one-way ANOVA followed by Tukey`s post-hoc test. H) *Genotype and treatment comparison* was performed by collapsing the data displayed in figures B), D) and F) according to each experimental group (Sal, LPS-1T and LPS-5T) and analyzed by one-way ANOVA followed by Tukey`s post-hoc test. Numbers in brackets refer to the total number of mice per each experimental group. Error bars represent the standard errors. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. 0 month; ^$p<0.05$, ^^$p<0.001$ vs. WT.

After nine months mice were subjected to the stepping test in order to assess other features of locomotor impairment such as forelimb akinesia, represented by the prolonged time needed to initiate a step, which can be caused by dysregulation of the dopamine system [456] and/or dopamine depletion [457]. The number of adjusted steps mice make with forepaws when lightly suspended by the tail and dragged across table surface for a length of one meter is used as an indication of akinesia.

In the figure 3.3 (A, B and C), the effect of single and repeated LPS injections was assessed on total number of adjusted steps within three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively).

As it may be observed in figure 3.3 A, single and repeated LPS injections decreased the number of adjusted steps, but not significantly, in WT mice. A similar trend was

also observed in five LPS-injected IL-1ra-/- mice (figure 3.3 C). However, single LPS injection did not induce any significant change in IL-1ra-/- mice (figure 3.3 C). In contrast, the ability of Casp-1-/- mice to initiate steps was not affected by single or repeated LPS inflammatory challenges (figure 3.3 B).

Treatment comparison among three different genotypes indicated that the ability of Casp-1-/- and IL-1ra-/- mice to adjust steps was significantly better versus WT mice (Sal, LPS-1T and LPS-5T) (figure 3.3 D). This difference was not due to the fact that WT mice displayed reduced ability to adjust steps as these animals had already shown to have similar performance at the age of eight months [457]. Therefore, the data suggests that better performance observed in Casp-1-/- and IL-1ra-/- was due to the inherent characteristics of these mouse strains.

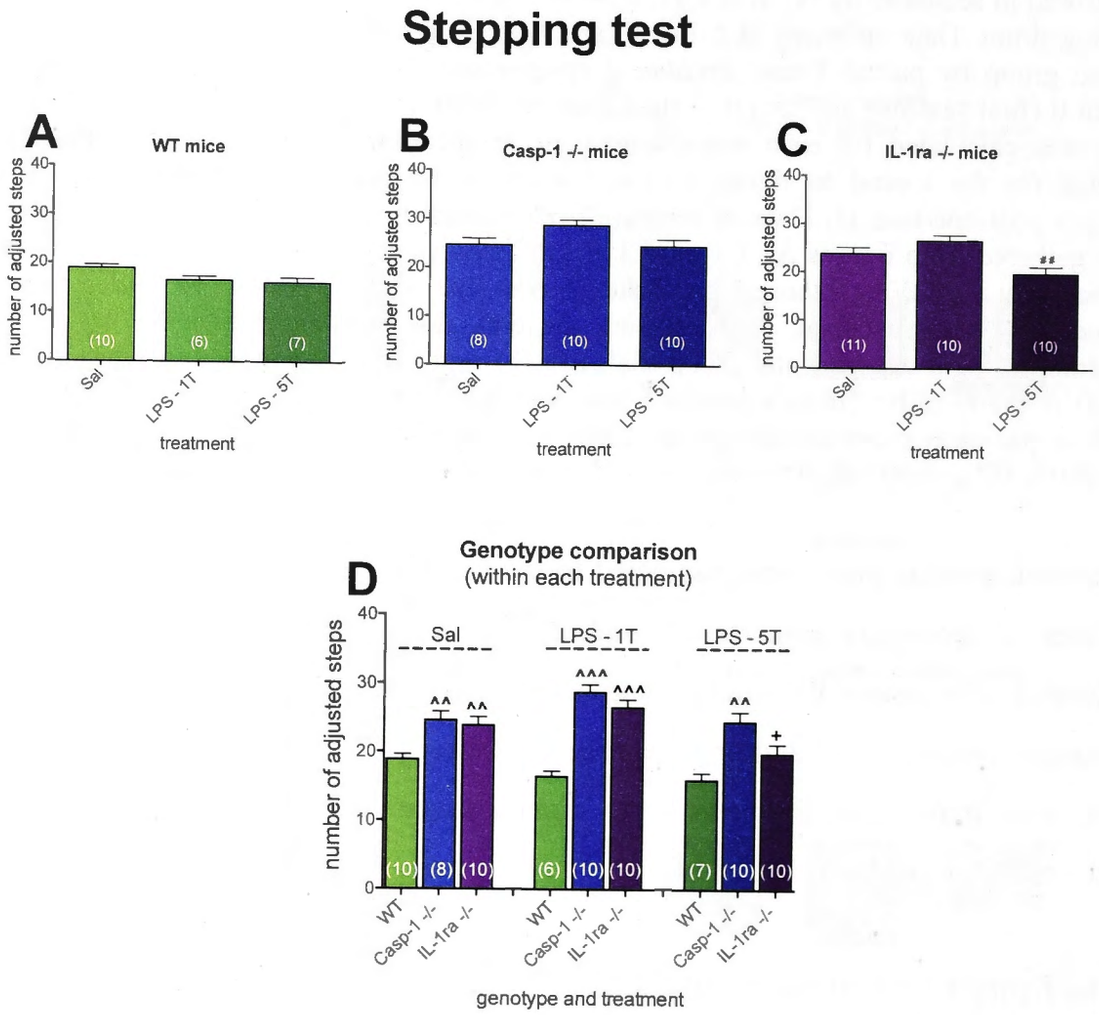


Figure 3.3: Long-term effect of LPS inflammatory challenge on mice stepping test ability: The number of adjusted steps was recorded as mean of three trials per animal and presented in seconds. The effect of LPS treatment was individualy assessed in A) WT, B) Casp-1-/- and C) IL-1ra-/- mice and analyzed by one-way ANOVA followed by Tukey`s post-hoc test. D) Data that were summarized from figures A), B) and C) were compared among different genotypes within same treatment. Results were analysed by one-way

ANOVA (Tukey`s multiple comparison test). $^{\#\#}p<0.001$ vs LPS-1T; $^{+}p<0.05$ vs. Casp-1-/-; ^^p<0.001, ^^^p<0.0001 vs. WT.

To determine the effect of LPS inflammatory challenge and its possible exacerbation by diet-induced obesity, the motor performance of single and five monthly LPS-injected WT mice fed a high-fat diet was assessed in the open-field and rotarod tests and compared with their controls (WT mice fed a regular diet).

As it is shown in figure 3.4 (A and C), there has not been observed significant decline in the open-field performance in neither RD no HFD-fed mice. Moreover, treatment comparison indicated that neither one nor repeated LPS injections altered the general performance of treated groups in comparison to their baseline (figure 3.4 B and D). Baseline data gathered at 0 month showed no differences in activity between RD and HFD-fed mice (figure 3.4 E). Overall comparisons between diets and treatments did not reveal any significant difference (figure 3.4 F).

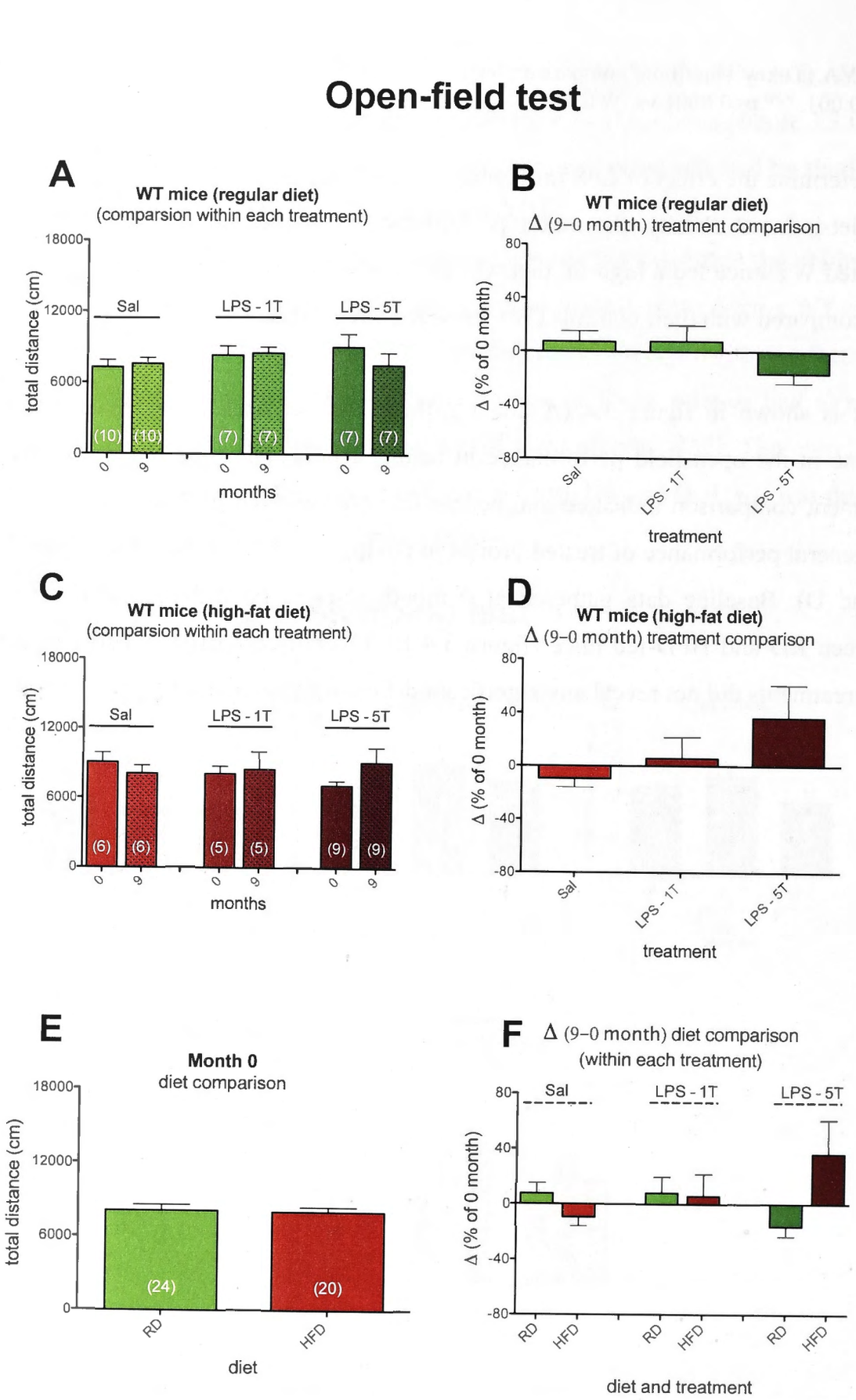


Figure 3.4: Effects of high-fat diet and LPS inflammatory challenge on total traveled distance during open-field test: *Comparison within each treated group*: The total distance travelled by A) RD and C) HFD-fed mice during 32 minutes before Sal or LPS injection (month 0) and after nine months starting from the first Sal/LPS injection (month 9). Data were presented as values of baseline points and session after nine months and analyzed by paired T-test. *Treatment comparison*: Percentage of difference between 0 month (first

session) and 9 month (last session) in B) RD and D) HFD-fed mice was calculated and expressed as delta. Results were analyzed by one-way ANOVA (Tukey's multiple comparison test) E) Data of *baseline performances* in the open-field test of two groups of mice fed with different diets were gathered from figures A) and C) and differences among them were compared by unpaired T-test. F) *Diet comparison within each treatment*: Comparison of motor activity between two groups of WT mice fed a different diet. Collected data were analyzed by unpaired T-test. Error bars represent standard errors.

Along with the open-field test, mice were also tested on the rotarod apparatus in order to investigate the long-term effects of high-fat consumption and LPS treatments on animal coordination.

As it was shown previously, mice fed a RD had experienced reduced ability to keep their balance on the rotarod apparatus. In saline-treated group of RD-fed mice, coordination declined by 19% ($p<0.05$) and in the single LPS-injected group, balance was reduced by 34% ($p<0.05$) (figure 3.5 A and B). A similar trend was observed in the five LPS-injected group of RD-fed mice, where decline in coordination was 22% but not statistically different versus baseline point (figure 3.5 A and B).

The reduced coordination in HFD-fed mice was not particularly affected by LPS treatment (figure 3.5 C and D). After nine months, the balance of saline-treated HFD mice on the rotarod was reduced by 62% ($p<0.01$) and a similar decline was observed with one LPS [68% ($p<0.05$)] and five LPS injections [60% ($p<0.0001$)] (figure 3.5 C and D).

Baseline performance of RD and HFD-fed mice in the rotarod test was not different (figure 3.5 E).

Overall, diet comparison within each treated group showed that the extent of coordination impairment observed in HFD-fed mice was markedly higher in comparison to RD-fed mice (figure 3.5 F).

Rotarod test

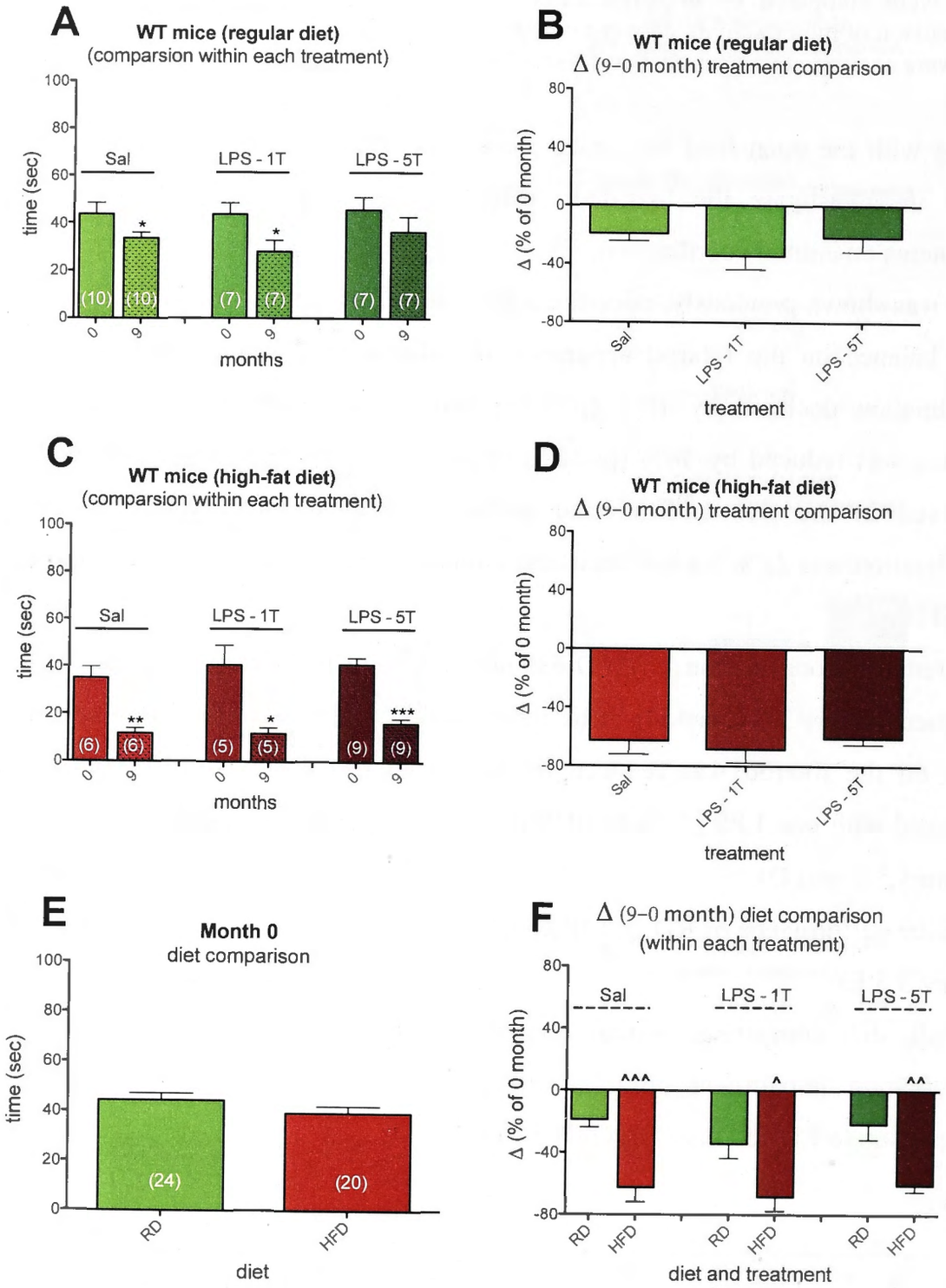


Figure 3.5: Effects of high-fat diet and peripheral LPS challenge on rotarod test performance: *Comparison within each treated group*: Average latency (measured in seconds) for A) RD and C) HFD-fed mice to fall down from the rotating drum was analyzed by paired T-test. *Treatment comparison*: Percentage of difference in the rotarod performance for time 0 and 9 months of B) RD and D) HFD-fed mice was calculated for each experimental group according to the previously described formula (figure 3.1) and compared by one-way ANOVA (Tukey`s multiple comparison test). E) Data of *baseline performances* of two groups of mice fed with different diets were gathered from figures A) and C) and

differences between them were analyzed by unpaired T-test F) *Diet comparison within each treatment*: Data displayed in figures B) and D) were collapsed according to each experimental group (Sal, LPS-1T and LPS-5T) and compared by unpaired T-test. Error bars represent the standard errors. *p<0.05, **p<0.01, ***p<0.0001 vs. 0 month; ^p<0.05, ^^p<0.01, ^^^p<0.001 vs. RD.

One of the first experiments performed after the open-field and rotarod test was the grid test, which gives reliable indications on the general condition of limb muscle strength [458]. The test was performed in order to exclude the possible effects of muscle strength disability that might occur in mice fed a HFD, which would have in turn affected their rotarod performance. General observation of the amount of time that animals spent hanging on the horizontally positioned grid indicated that muscle strength was not impaired with peripheral LPS treatment in the group of mice fed either RD (figure 3.6 A), or HFD (figure 3.6 B). Assessment of treatment effect between different diet-fed mice indicated that long-term consumption of high-fat diet did not impair muscle strength in mice (figure 3.6 C).

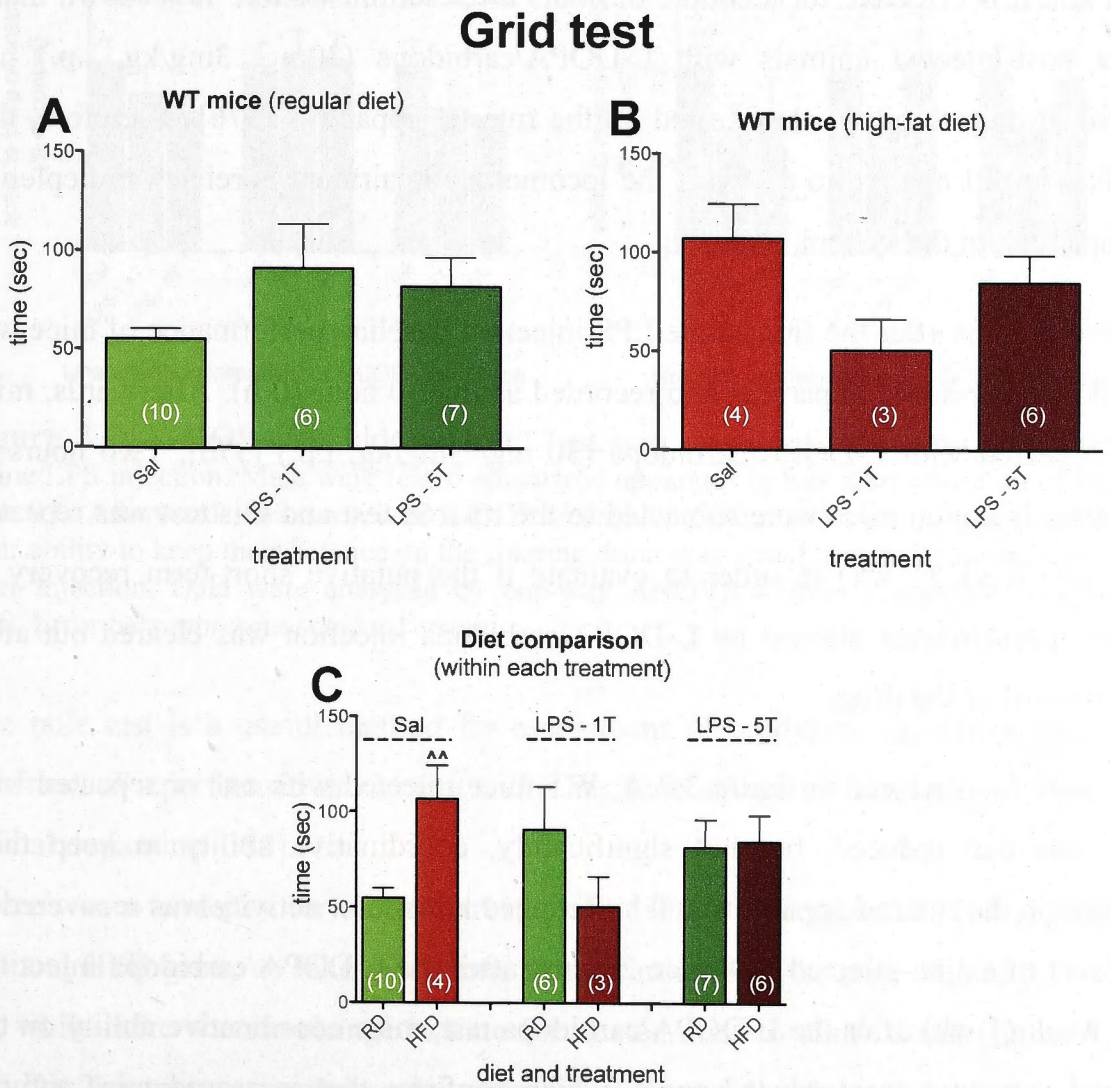


Figure 3.6: Mice performance in the grid test: Limb muscle strength of WT mice that

were injected with saline or LPS on grid apparatus. Latency for animal to fall from grid of the apparatus was recorded in seconds. Time that mice fed with either A) RD, or B) HFD spent hanging on the horizontally positioned metal grid was analyzed by one-way ANOVA (Tukey`s multiple comparison test). C) Gathered data from figures A) and B) were divided into two groups according to the treatment and compared by unpaired T-test. Error bars represent standard errors. ^^$p<0.01$ vs. RD.

In order to understand if the loss of locomotor activity observed in the rotarod test after nine months from the first saline/LPS injection was related to impairment in nigrostriatal dopamine activity, mice were subjected to L-DOPA/carbidopa test. L-DOPA/carbidopa is a combination of drugs given to Parkinson`s diseased patients [459, 460], as a replacement for the lack of dopamine. L-DOPA is a precursor of neurotransmitter dopamine and therefore it is used to increase dopamine concentration in the treatment of Parkinson`s disease. Carbidopa is a drug given in combination with L-DOPA in order to inhibit peripheral metabolism of L-DOPA to dopamine since dopamine itself is not able to cross BBB. The effect of this drug is short and it is effective for a couple of hours after administration. It is shown that 2 hours post-injected animals with L-DOPA/carbidopa (30mg/ 3mg/kg, i.p.) had improved motor ability when tested on the rotarod apparatus [376]. Therefore, this test is a useful method to assess if the locomotor impairment is related to depletion of dopamine in the system.

Twelve months after the first saline/LPS injection baseline performance of mice was tested on the rotarod apparatus and recorded as time 0 hour (0 h). Afterwards, mice were injected with L-DOPA/carbidopa (30 mg/ 3mg/kg, i.p.) [376]. Two hours (2 hrs) after injection mice were subjected to the rotarod test and this test was repeated after one week (1 wk) in order to evaluate if the putative short-term recovery in rotarod performance elicited by L-DOPA/carbidopa injection was cleared out after the retrieval of the drug.

As it may be observed in figure 3.7 A, WT mice injected with one or repeated LPS injections had reduced, but not significantly, coordinative ability to keep their balance on the rotarod apparatus at 0 h. Reduced locomotor activity was recovered to the level of saline-injected RD mice 2 hours after the L-DOPA/carbidopa injection. One week (1 wk) after the L-DOPA/carbidopa test, mice coordinative ability on the rotarod apparatus was reduced again, which confirms that increased mice activity

after 2 hours post injection was due to the lack of dopamine in the nigrostriatal system.

High-fat diet-fed mice had heterogenous response to L-DOPA/carbidopa test. As it is presented in figure 3.7 B reduced activity in HFD-fed mice that received one or five LPS injections was not recovered to the same extent as it was observed in RD-fed mice two hours after L-DOPA/carbidopa injection. Single LPS-injected HFD-fed mice did not respond to a L-DOPA/carbidopa test, whereas there was positive response in five LPS-injected mice, but the drug effect was not completely cleared out after one week.

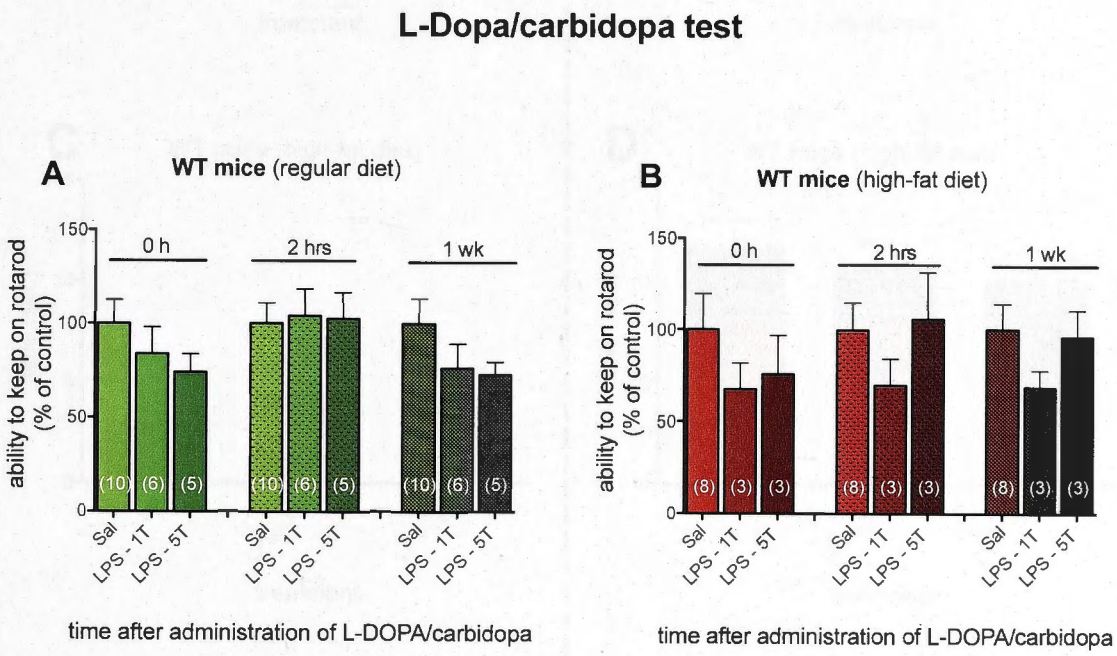


Figure 3.7: L-DOPA/carbidopa test: Test was conducted 12 months after the first saline/LPS injection. Mice were tested on rotarod apparatus before administration of drug at time 0 h. Afterwards, mice received L-DOPA/carbidopa (30 mg/ 3mg/kg, i.p.) injection and their ability to keep their balance on the rotating drum was tested in next 2 hrs and one week after injection. Data were analyzed by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors.

The pole test is a useful method for assessment of bradykinesia, which describes slowness of a performed movement and it has been shown to correlate with levels of striatal dopamine [388].

There was a tendency for animals treated with single or repeated LPS injections in RD and HFD group to take more time to turn around on the pole (figure 3.8 A and C), while there was no change in time for treated mice to climb down (figure 3.8 B and D). Treatment comparison between two different diet-fed groups indicated that HFD-fed mice tended to spend more time to turn around on the pole accompanied

with significant delay to climb down (figure 3.8 E and F). These data provided from the pole test suggest that the level of dopaminergic neurotransmission might be impaired in HFD-fed mice that could have finally resulted in slowness of movements.

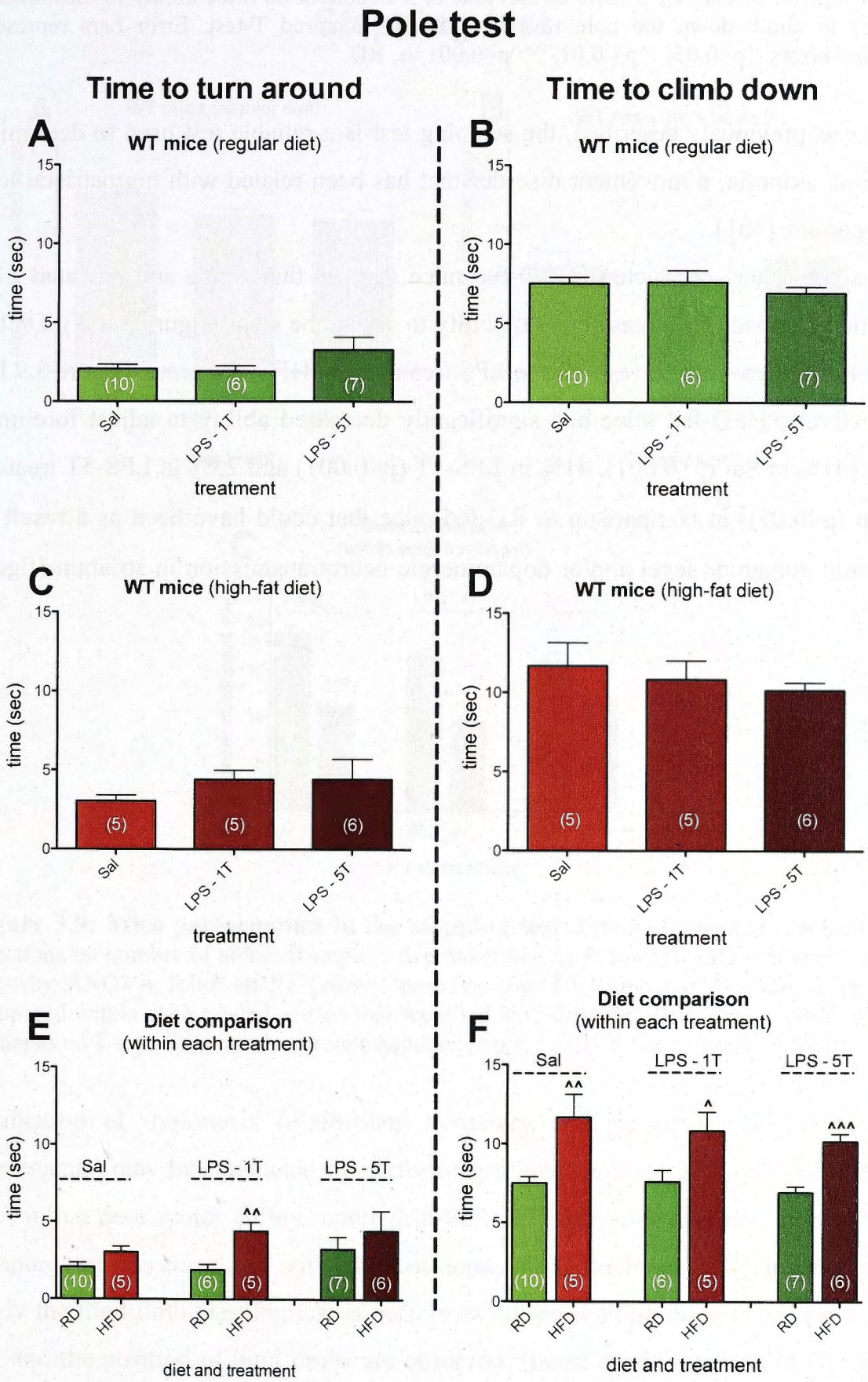


Figure 3.8: Effects of high-fat diet and LPS inflammatory challenge on pole test performance: Latency for WT mice to turn around and climb down the pole was recorded in seconds. Time that mice fed either A) RD, or C) HFD spent to turn around the pole was analyzed by one-way ANOVA followed by Tukey's post-hoc test. Latency of B) RD or D) HFD-fed mice to climb down the pole was analyzed by one-way ANOVA followed by

Tukey`s post-hoc test. E) Effects of diet and LPS treatment on mice ability to turn around and F) to climb down the pole was analyzed by unpaired T-test. Error bars represent standard errors. ^p<0.05, ^^p< 0.01, ^^^p<0.001 vs. RD.

As it was previously described, the stepping test is a reliable test used to determine forelimb akinesia, a movement disorder that has been related with nigrostriatal loss of dopamine [461].

The stepping test conducted in RD-fed mice showed that single and repeated LPS injections tended to decrease animal ability to adjust the steps (figure 3.9 A). On the other hand, there was no response to LPS treatment in HFD-fed group (figure 3.9 B). Collectively, HFD-fed mice had significantly decreased ability to adjust forelimbs steps [41% in Sal ($p<0.001$), 41% in LPS-1T ($p<0.001$) and 25% in LPS-5T treated-group ($p<0.05$)] in comparison to RD-fed mice that could have been as a result of impaired dopamine level and/or dopaminergic neurotransmission in striatum (figure 3.9 C).

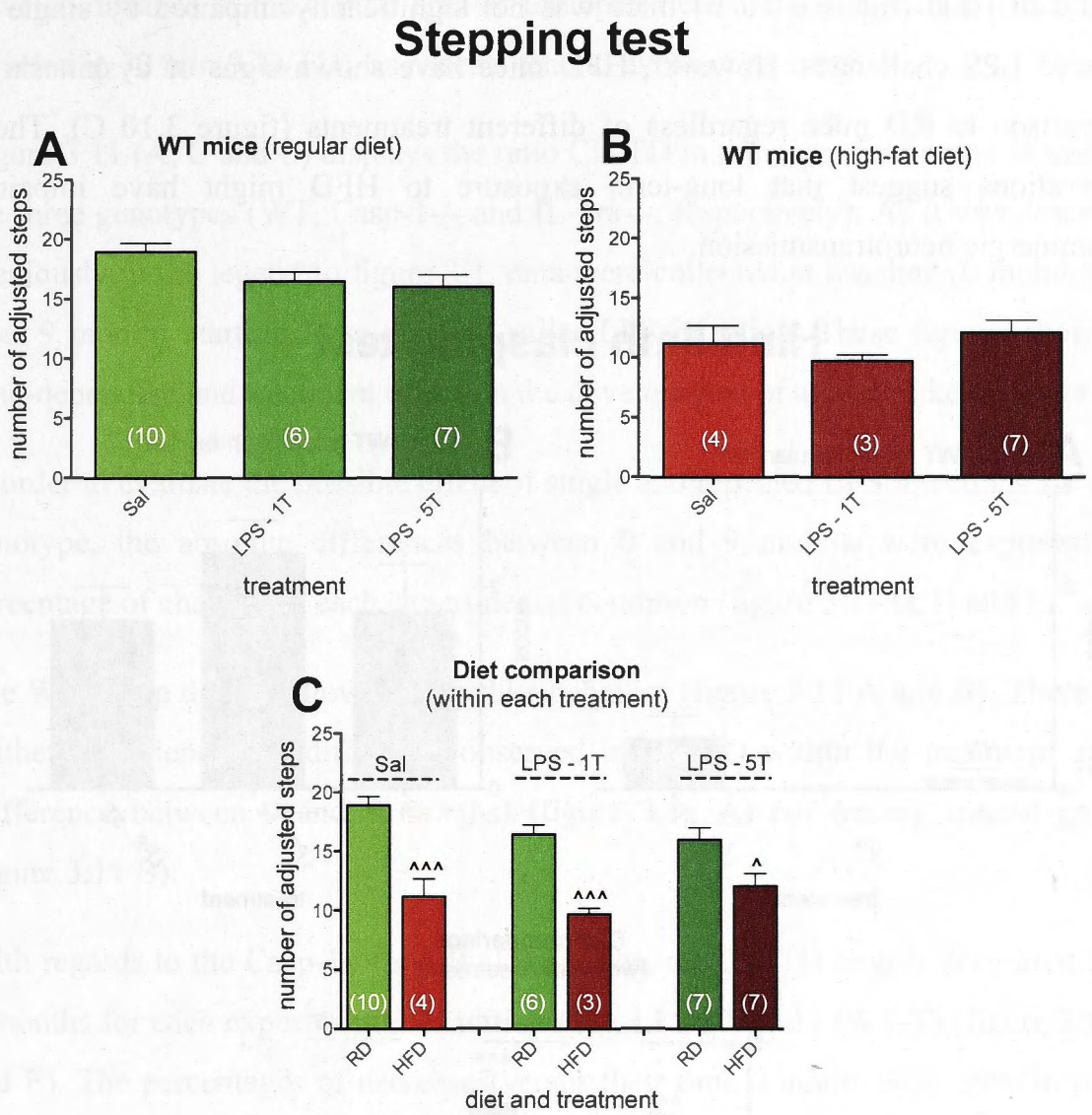


Figure 3.9: Mice performance in the stepping test: Effect of single or repeated LPS injections on number of adjusted step in mice fed either A) RD or B) HFD was analyzed by one-way ANOVA followed by Tukey`s post-hoc test. C) Number of adjusted steps was compared within each treated groups that were fed with different diets. Data were analyzed by unpaired T-test. Error bars represent standard errors. ^p<0.05, ^^^p<0.001 vs. RD.

Evaluation of dyskinesia (diminished voluntary and prevalence of involuntary movements) may be conducted by performing a hind limb clasping test. It has been shown that poor motor ability reported in BH_4-deficient mice assessed in hind limb clasping test was correlated with a lack of dopamine in the brain [462]. In the current study the hind limb clasping test is performed by suspending mice in the air by the tail and the position of hind limbs are observed. Based on the position of the limbs mice are scored a 0 (hind limbs are splayed outwards), 1 (one hind limb is retracted towards abdomen), 2 (both hind limbs are partially retracted towards abdomen) and 3 (hind limbs are completely retracted and touching the abdomen). Results gathered from hind limb clasping test indicate that neuromuscular function in RD (figure 3.10

A) and in HFD (figure 3.10 B) mice was not significantly impaired by single or repeated LPS challenges. However, HFD mice have shown signs of dyskinesia in comparison to RD mice regardless of different treatments (figure 3.10 C). These observations suggest that long-term exposure to HFD might have impaired dopaminergic neurotransmission.

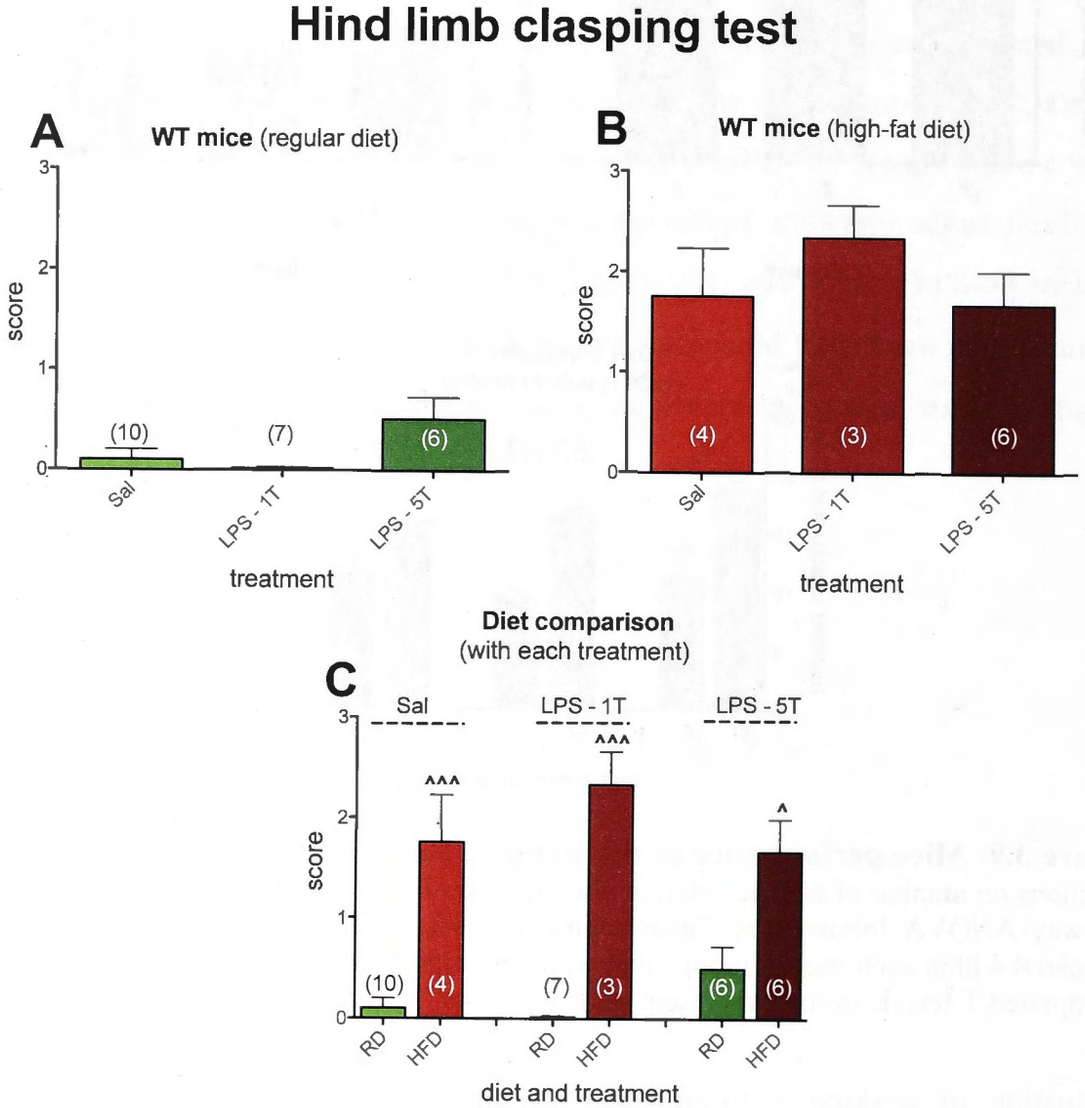


Figure 3.10: Hind limb clasping test: Wild type mice were suspended in the air for 10 seconds by the tail and their performance to clasp hind limbs was scored. Score for hind limb clasping test of WT mice fed either A) RD or B) HFD was analyzed by one-way ANOVA (Tukey`s post-hoc test). C) *Diet comparison within each treatment*: Data were analyzed by unpaired T-test. Error bars represent standard error. ^p< 0.05, ^^^p<0.001 vs. RD.

3.4.2 Evaluation of an anxiety/depressive-like behavior

As it was described previously, inflammation induced dysregulation in nigrostriatal signaling pathways may also lead to anxiety/depressive-like behavior. One of the parameters used for the assessment of anxiety-like behavior is the ratio between

traveled distance in the central area of the open-field arena and total distance mice traveled in 32 min (CD/TD), as rodents tend to avoid open areas.

Figure 3.11 (A, C and E) displays the ratio CD/TD in the open-field arena of mice of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively). As it was described previously in the legend to figure 3.1, data were collected at baseline (0 month) and after 9 months starting from the first saline/LPS injection. These figures show the time-dependent and treatment effect on the development of anxiety-like behavior.

In order to evaluate the possible effect of single and repeated LPS injections for each genotype, the absolute differences between 0 and 9 months were expressed as percentage of change for each experimental condition (figure 3.11 B, D and F).

The WT group did not show anxiety-like behavior (figure 3.11 A and B). There was neither time-dependent difference observed in CD/TD within the treatment group (difference between 0 and 9 months) (figure 3.11 A) nor among treated groups (figure 3.11 B).

With regards to the Casp-1-/- and IL-1ra-/- mice, the CD/TD largely decreased after 9 months for each experimental condition (Sal, LPS-1T and LPS 5-T) (figure 3.11 C and E). The percentages of decreases versus their time 0 month were 39% in saline ($p<0.01$), 57% in LPS-1T ($p<0.01$) and 42% in LPS-5T ($p<0.01$) treated Casp-1-/- mice (figure 3.11 C). The comparison among experimental groups did not reveal any significant differences (figure 3.11 D). In a similar manner, the percentages of difference after 9 months in IL-1ra-/- mice was 72% in saline ($p<0.001$), 52% in LPS-1T ($p<0.14$) and 73% in LPS-5T ($p<0.001$) (figure 3.11 E) with no evident differences among experimental groups (figure 3.11 F). These data suggest that Casp-1-/- and IL-1ra-/- mice displayed anxiety-like behavior that was not additionally exacerbated by LPS administration.

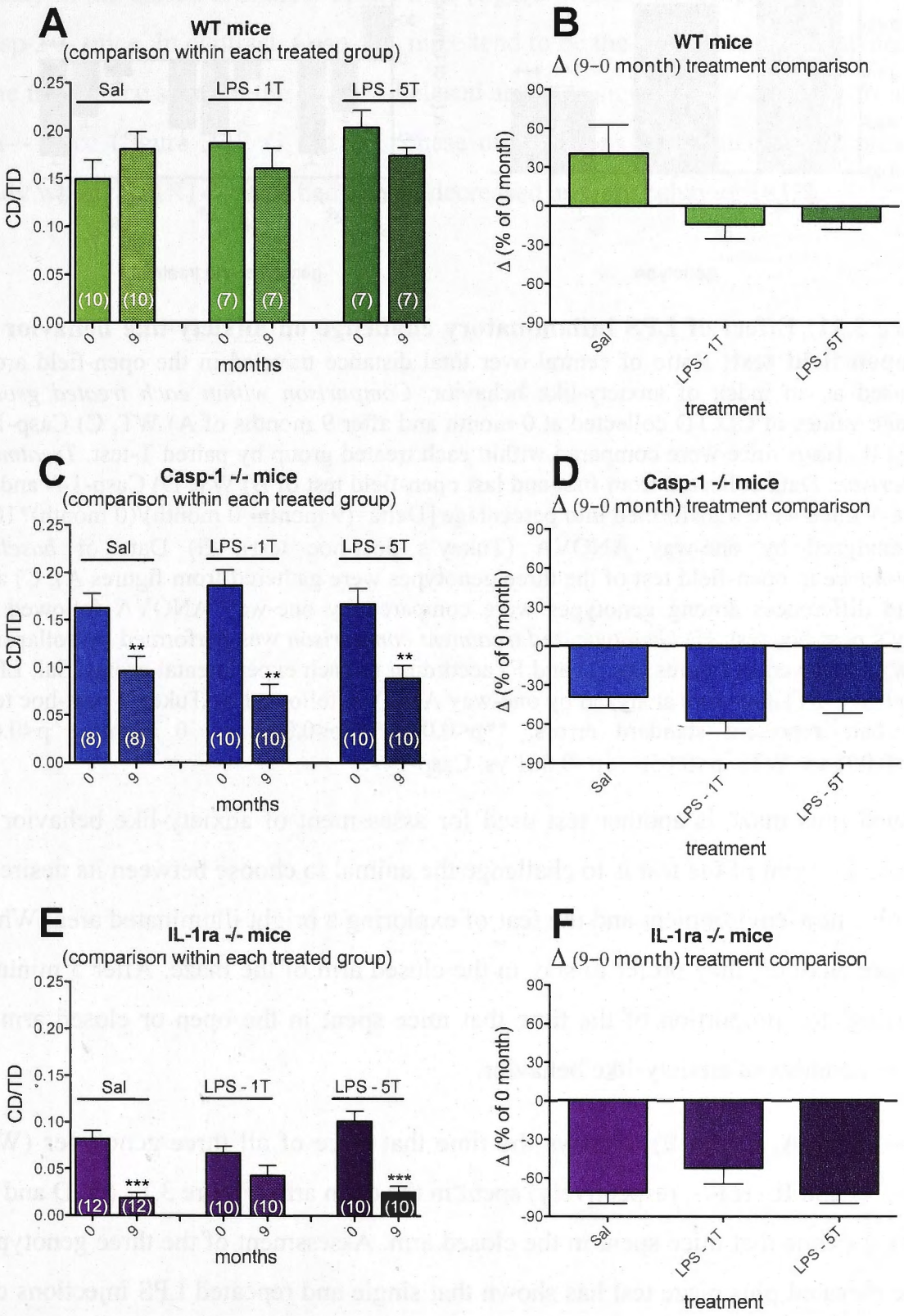
Figures 3.11 G and H were constructed to evaluate the genotype differences for each experimental group at baseline (time 0 month) (figure 3.11 G) and after 9 months (figure 3.11 H) as it was previously described in figure 3.1.

Figure 3.11 G shows that there were significant differences among the three genotypes at baseline. The CD/TD ratio of IL-1ra-/- mice was significantly lower by 53% versus WT ($p<0.001$) and 52% lower in comparison to Casp-1-/- mice

($p<0.001$), suggesting that IL-1ra-/- mice displayed anxiety-like behavior when mice were very young, 42 days old.

The percentages of changes that were previously calculated for the individual strains (figure 3.11 B, D and F) were gathered in figure 3.1 H and compared among genotypes within each treatment. Despite the fact that IL-1ra-/- mice displayed nearly 2-fold lower CD/TD than both WT and Casp-1-/- mice at 0 month (figure 3.11 G), their CD/TD significantly decreased at 9 months in the three treated groups (Sal, LPS-1T and LPS-5T) by approximately 60% (figure 3.11 H). Casp-1-/- mice showed a trend to be more anxious than WT mice and it reached significance in one and 5-times LPS-injected groups. However, the level of anxiety-like behavior in IL-1ra-/- mice was higher, but not statistically different, in comparison to Casp-1-/- mice.

Open-field test (central/total distance)



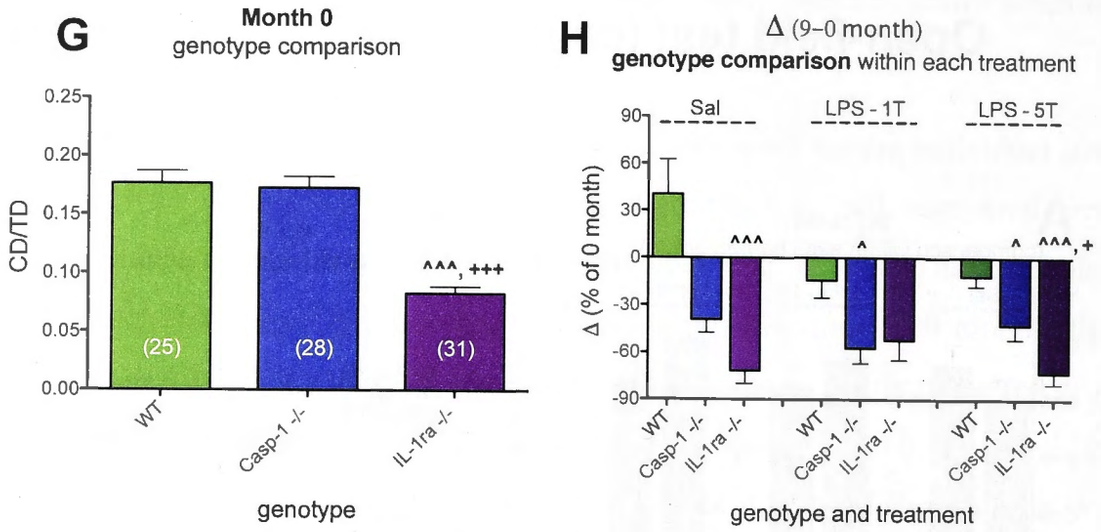


Figure 3.11: Effect of LPS inflammatory challenge on anxiety-like behavior in the open-field test: Ratio of central over total distance traveled in the open-field arena was used as an index of anxiety-like behavior. *Comparison within each treated group*: Average values of CD/TD collected at 0 month and after 9 months of A) WT, C) Casp-1-/- and E) IL-1ra-/- mice were compared within each treated group by paired T-test. *Treatment comparison*: Data collected from first and last open-field test of B) WT, D) Casp-1-/- and F) IL-1ra-/- mice were transformed into percentage [Delta=(9 month- 0 month)/(0 month)*100] and analyzed by one-way ANOVA (Tukey`s post-hoc test). G) Data of *baseline performance* in open-field test of the three genotypes were gathered from figures A), C) and E) and differences among genotypes were compared by one-way ANOVA followed by Tukey`s post-hoc test. H) *Genotype and treatment comparison* was performed by collapsing the data displayed in figures B), D) and F) according to each experimental group (Sal, LPS-1T and LPS-5T) and were analyzed by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. **p<0.01, ***p<0.001 vs. 0 month; ^p<0.05, ^^^p<0.001 vs. WT; $^{+}$p<0.05, $^{+++}$p<0.001 vs. Casp-1-/-.

Elevated plus maze is another test used for assessment of anxiety-like behavior in rodents. The aim of the test is to challenge the animal to choose between its desire to explore a new environment and the fear of exploring a bright illuminated area. When mice are anxious, they prefer to stay in the closed arm of the maze. After 5 minutes of testing, the proportion of the time that mice spent in the open or closed arm is used as an index of anxiety-like behavior.

Figure 3.12 (A, C and E) displays the time that mice of all three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) spent in the open arm. Figure 3.12 (B, D and F) shows the time that mice spent in the closed arm. Assessment of the three genotypes in the elevated plus maze test has shown that single and repeated LPS injections did not further exacerbate the anxiety-like behavior in comparison to their own saline-treated group of mice.

On the other hand, genotype comparison has revealed that IL-1ra-/- mice were the group that showed clear signs of increased anxiety since these animals had spent significantly less time in the open arm (figures 3.12 G) and concomitantly preferred to stay in the closed arm most of the time (figure 3.12 H) in comparison to WT and Casp-1-/- mice. In contrast, Casp-1-/- mice tend to be the least stressed group and the time these mice spent in the open and closed arm was significantly different from IL-1ra-/- mice (figure 3.12 G and H). These observations are related to the previous study where IL-1R1-/- mice had shown decreased anxiety behavior [431].

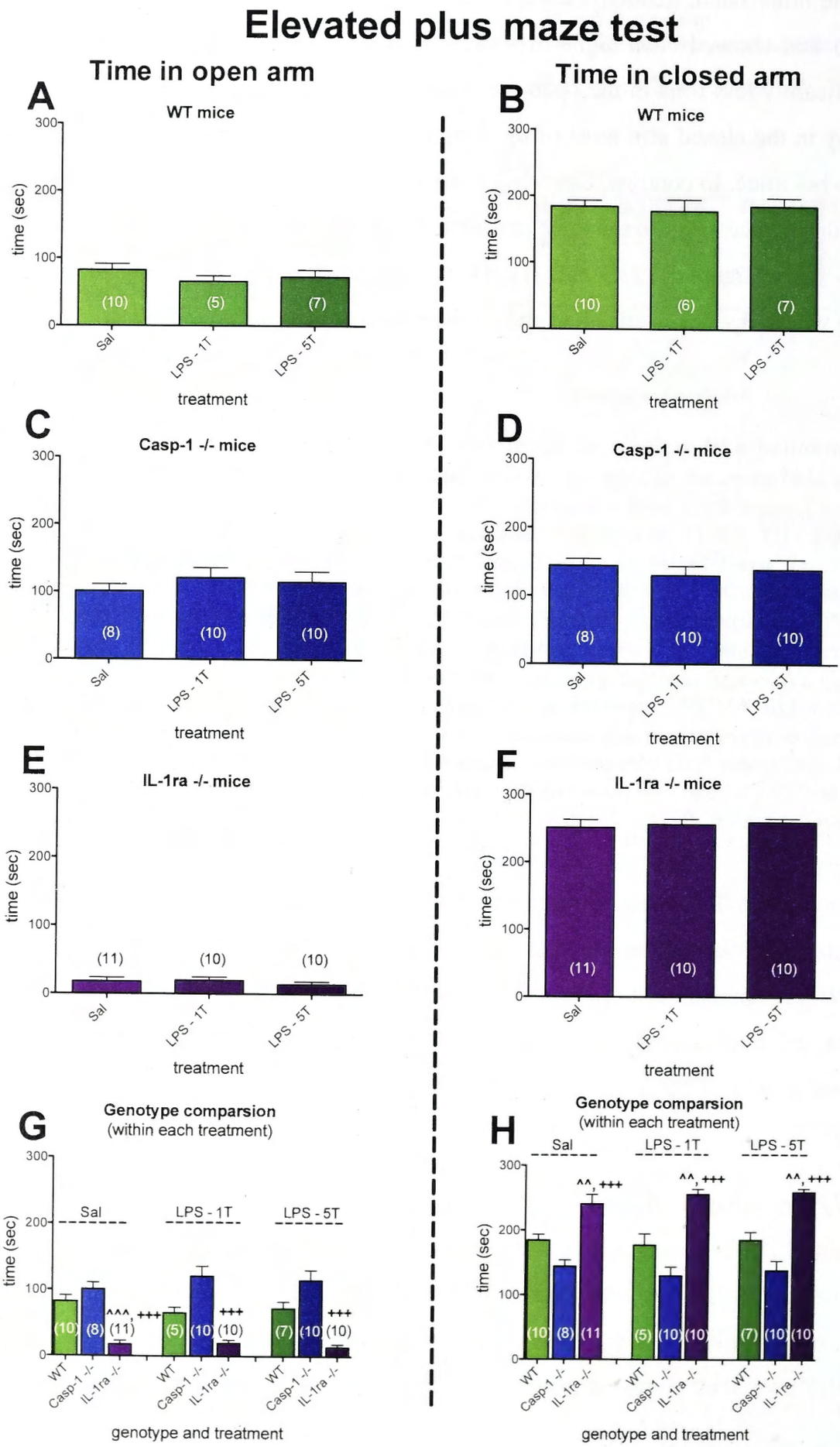


Figure 3.12: Effect of the LPS inflammatory challenge on anxiety-like behavior in the elevated plus maze test: The proportion of time (5 min in total) that mice spent in

open or closed arm of elevated plus maze apparatus was measured in seconds. *Treatment comparison*: Time that A) WT, C) Casp-1-/- and E) IL-1ra-/- mice spent in open arm and B) WT, D) Casp-1-/- and F) IL-1ra-/- in closed arm, respectively. Differences within each genotype were analyzed by one-way ANOVA followed by Tukey`s post-hoc test. *Genotype comparison* within each treatment in open G) and closed arm H) was analyzed by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors. ^^p<0.001, ^^^p<0.0001 vs. WT, $^{+++}$p<0.0001 vs. Casp-1-/-.

The forced swimming test is frequently used to assess depressive-like behavior in rodents [463, 464]. Mice are placed individually in a cylinder half-filled with water to prevent them from escape during the test. Therefore, mice are forced to stay for five minutes in the water. The time that mice spend active (swimming and struggling) or passive (floating) is measured in seconds. The proportion of time that mice spend passive (floating) is considered to reflect the behavioral despair in animal, which results from the animal`s sense of hopelessness to persist in its attempt to find an escape.

The role of IL-1 signaling pathway and LPS inflammatory challenge on depressive-like behavior in WT, Casp-1-/- and IL-1ra-/- mice was analyzed and presented in the figure 3.13. The group of single-injected IL-1ra-/- mice was not tested since there was no significant difference between saline and LPS-5T treated groups.

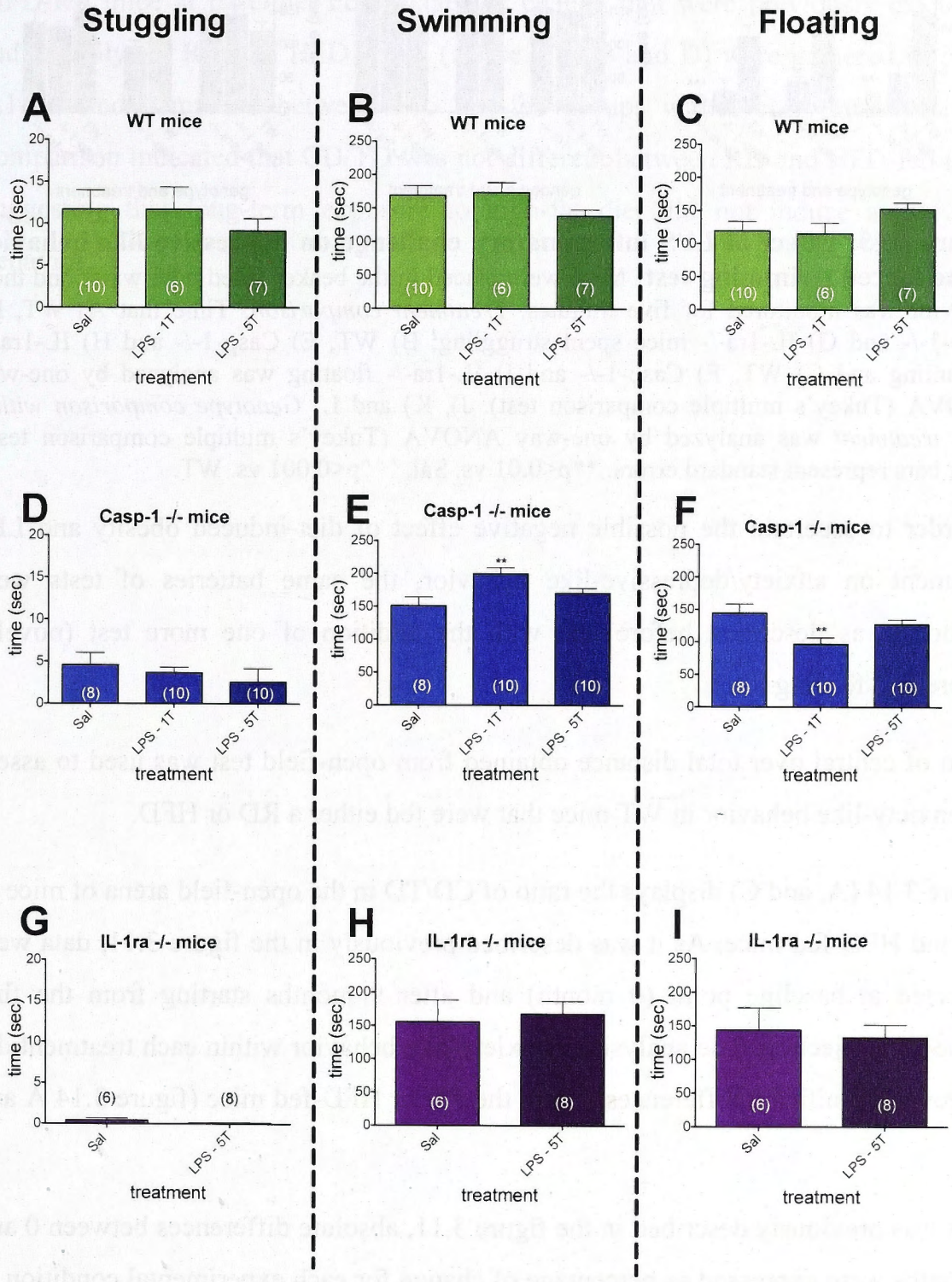
As it is shown in figure 3.13 (A, B and C), the total time that WT mice, injected with five LPS injections, spent struggling (figure 3.13 A), and swimming (figure 3.13 B) was reduced but not significantly, whereas floating time tended to increase (figure 3.13 C). Single LPS injection did not alter the performance of WT mice in the forced swimming test (figure 3.13 A, B and C). These results suggest that repeated LPS challenges showed a trend to induce depressive-like behavior in WT mice, but the results were not significant.

In regard to Casp-1-/- mice, single and 5-times LPS-injected mice had increased swimming time and reduced floating time (figure 3.13 E and F). Struggling time was not affected by LPS challenge (figure 3.13 D). These observations imply that LPS inflammatory challenge did not induce or exacerbate the depressive-like behavior in Casp-1-/- mice.

Similarly to the Casp-1-/- mice, repeated LPS inflammatory challenges did not alter the time that IL-1ra-/- spent struggling, swimming and floating (figure 3.13 G, H and I).

Genotype comparison (WT, Casp-1-/- and IL-1-ra -/-) and within each treatment revealed that IL-1ra-/- mice spent significantly less time struggling in comparison to WT mice (figure 3.13 J). On the other hand, there was no difference among genotypes in swimming and floating (figure 3.13 K and L). Although struggling was significantly lower in IL-1ra-/- mice, floating time was similar to other two genotypes, so it might be suggested that IL-1ra-/- mice did not show depressive-like behavior.

Forced swimming test



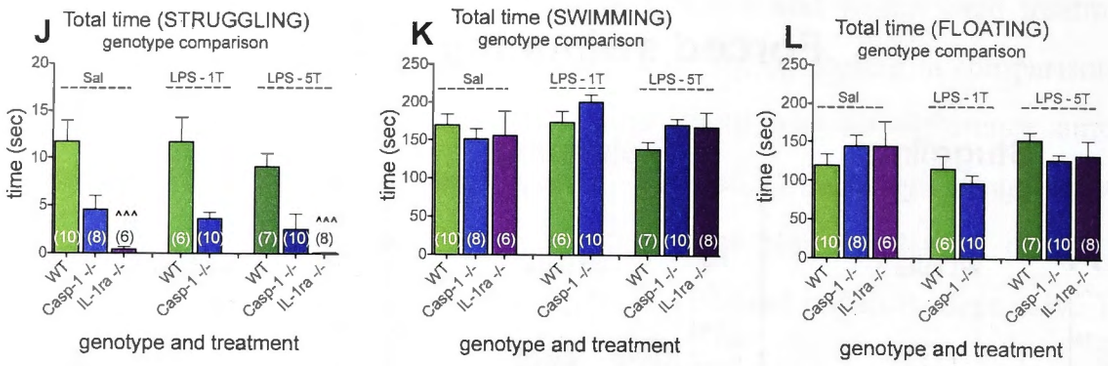


Figure 3.13: Effect of LPS inflammatory challenge on depressive-like behavior in the forced swimming test: Mice were placed in the beaker filled with water and their behavior was monitored for five minutes. *Treatment comparison*: Time that A) WT, D) Casp-1-/- and G) IL-1ra-/- mice spent struggling; B) WT, E) Casp-1-/- and H) IL-1ra-/- swimming and C) WT, F) Casp-1-/- and I) IL-1ra-/- floating was analyzed by one-way ANOVA (Tukey`s multiple comparison test). J), K) and L) *Genotype comparison within each treatment* was analyzed by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors. **p<0.01 vs. Sal, ^^^p<0.001 vs. WT.

In order to ascertain the possible negative effect of diet-induced obesity and LPS treatment on anxiety/depressive-like behavior, the same batteries of tests were performed as described before and with the addition of one more test (novelty suppressed feeding test).

Ratio of central over total distance obtained from open-field test was used to assess the anxiety-like behavior in WT mice that were fed either a RD or HFD.

Figure 3.14 (A, and C) displays the ratio of CD/TD in the open-field arena of mice of RD and HFD-fed mice. As it was described previously in the figure 3.11, data were collected at baseline point (0 month) and after 9 months starting from the first saline/LPS injection. The analysis of anxiety-like behavior within each treatment did not reveal significant differences within the RD or HFD-fed mice (figure 3.14 A and C).

As it was previously described in the figure 3.11, absolute differences between 0 and 9 months were expressed as percentage of change for each experimental condition in order to assess the effect of single and repeated LPS inflammatory challenges on anxiety-like behavior (figure 3.14 B and D). As it is presented in figures 3.14 B and D, there was no treatment effect in RD and HFD-fed mice, suggesting the LPS treatment did not induce or exacerbate anxiety-like behavior in both groups of mice.

Differences between the two diet-fed groups are evaluated in figure 3.14 (E and F). Figure 3.14 E indicates that there were not significant differences between RD and HFD-fed mice at baseline. Percentages of change that were previously calculated individually for RD and HFD group (figure 3.14 B and D) were gathered in figure 3.14 F and compared between two diet-fed groups within each treatment. The comparison indicated that CD/TD was not different between RD and HFD-fed mice, suggesting that long-term exposure to high-fat diet did not induce anxiety-like behavior.

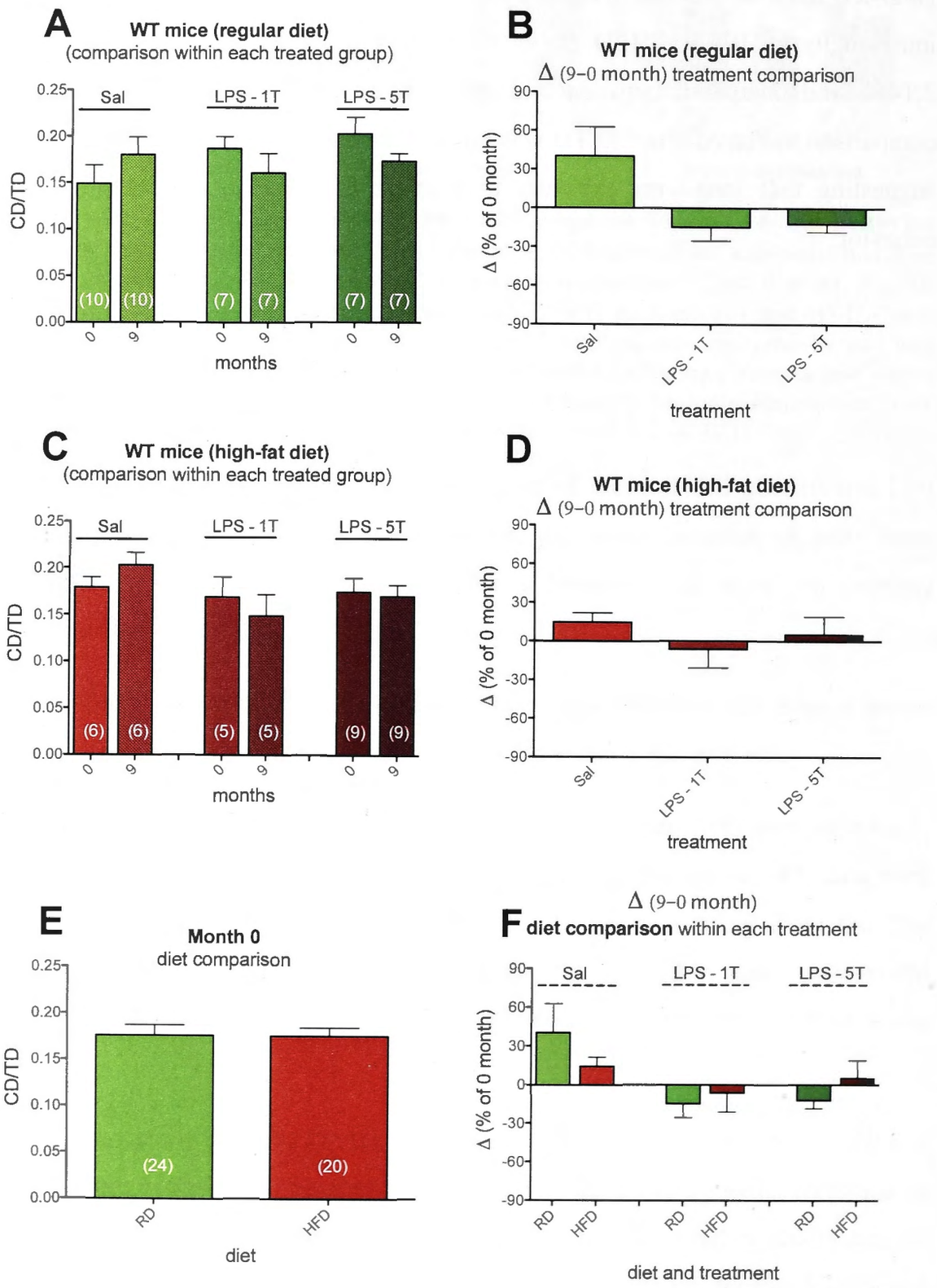


Figure 3.14: Effects of LPS inflammatory challenge and high-fat diet on anxiety-like behavior in the open-field test: Ratio of central over total distance traveled in open-field arena was used as an index of anxiety-like behavior. *Comparison within each treated group*: Average values of CD/TD collected at 0 month and after 9 months in A) RD and C) HFD-fed mice were compared by paired T-test. *Treatment comparison*: Data collected from first and last open-field test in B) RD and D) HFD-fed

mice were transformed into percentages [Delta=(9 month- 0 month)/(0 month)*100] and analyzed by one-way ANOVA (Tukey`s multiple comparison test) E) Data of *baseline performances* of two groups of mice were gathered from figures A) and C) and differences between them were compared by unpaired T-test F) *Diet comparison within each treatment* was performed by collapsing the data displayed in figures B) and D) according to each experimental group (Sal, LPS-1T and LPS-5T) and analyzed by unpaired T-test. Error bars represent standard errors.

Anxiety-like behavior was also assessed in the elevated plus maze test. Figure 3.15 (A and C) displays the time that RD and HFD-fed mice spent in the open arm. In figure 3.15 (B and D) the time mice spent in the closed arm is shown. Assessment of both RD and HFD-fed mice revealed that single and repeated LPS injections did not induce or exacerbate anxiety-like behavior in these mice.

Comparison between the two different diet-fed groups (RD and HFD) within same treatment indicated that long-term consumption of HFD did not induce anxiety-like behavior (figure 3.15 E and F).

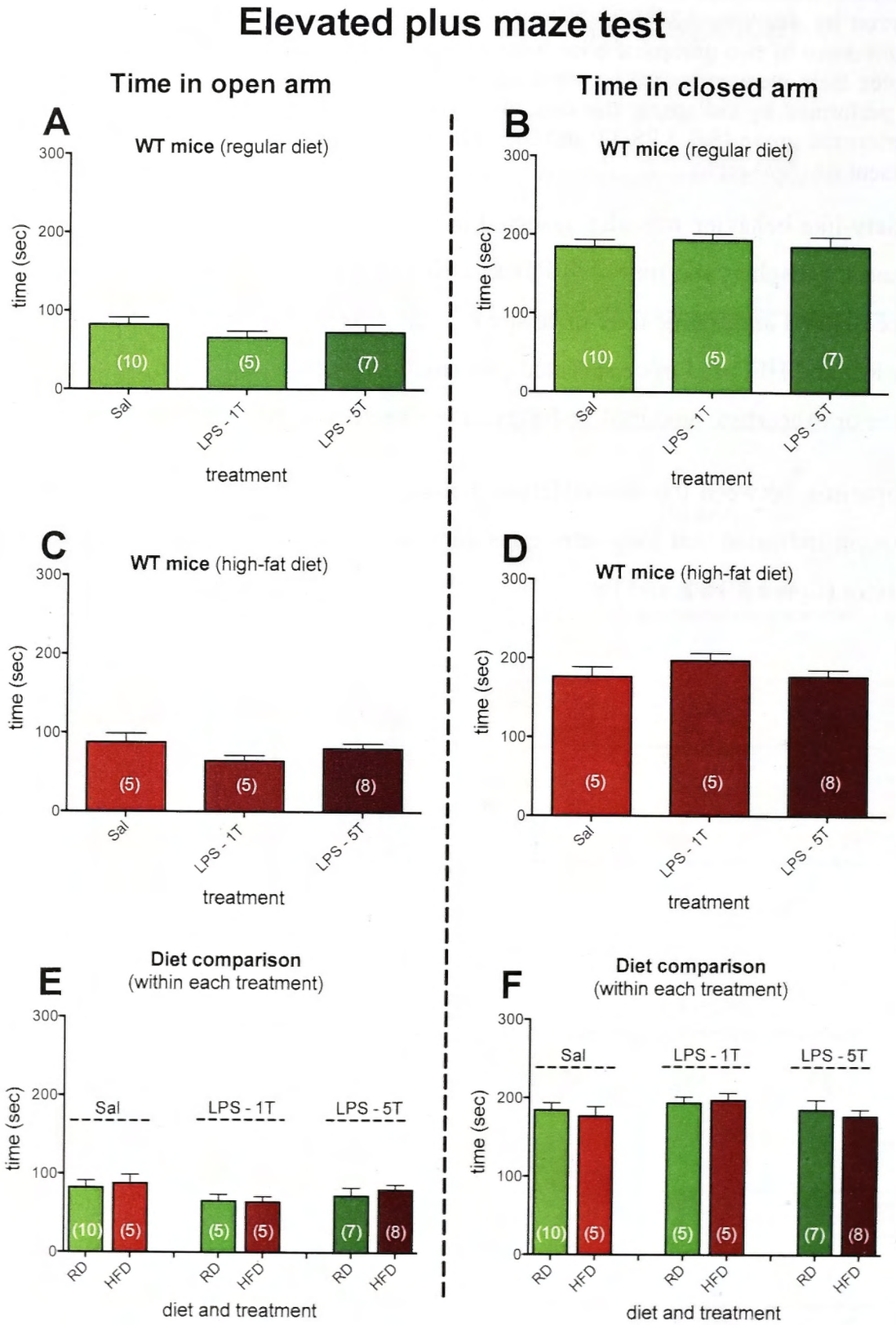


Figure 3.15: Effects of LPS inflammatory challenge and high-fat diet on anxiety-like behavior in the elevated plus maze test: The proportion of time (5 min in total) that mice spent in open or closed arm of elevated plus maze apparatus was measured in seconds. *Treatment comparison*: Time that A) RD and C) HFD-fed mice spent in open arm, or B) RD and D) HFD in closed arm were analyzed by one-way ANOVA (Tukey's multiple comparison test). *Diet comparison*: Time mice spent in E) open arm or F) closed arm was compared between two diet-fed groups within same treatment and analyzed by unpaired T-test. Error bars represent standard errors.

In order to assess the effects of high-fat diet and LPS treatment on depressive-like behavior, mice were subjected to forced swimming test.

As it was described before, 5-times LPS-injected RD-fed mice displayed a tendency to struggle (figure 3.16 A) and swim (figure 3.16 B) less, whereas floating time tended to be increased (figure 3.16 C). Overall, these results suggest that repeated LPS challenges tended to induce depressive-like behavior in RD mice, but the results were not significant.

As shown in figure 3.16 (D, E and F) neither high-fat diet nor single and repeated LPS inflammatory challenge altered the time that HFD-fed mice spent struggling, swimming and floating.

Comparison between two diet-fed groups (figure 3.16 G, H and I) and within each treatment suggested that HFD-fed mice showed a tendency to struggle less, swim more and spend less time floating. These data suggest that long-term high-fat diet consumption did not induce depressive-like behavior.

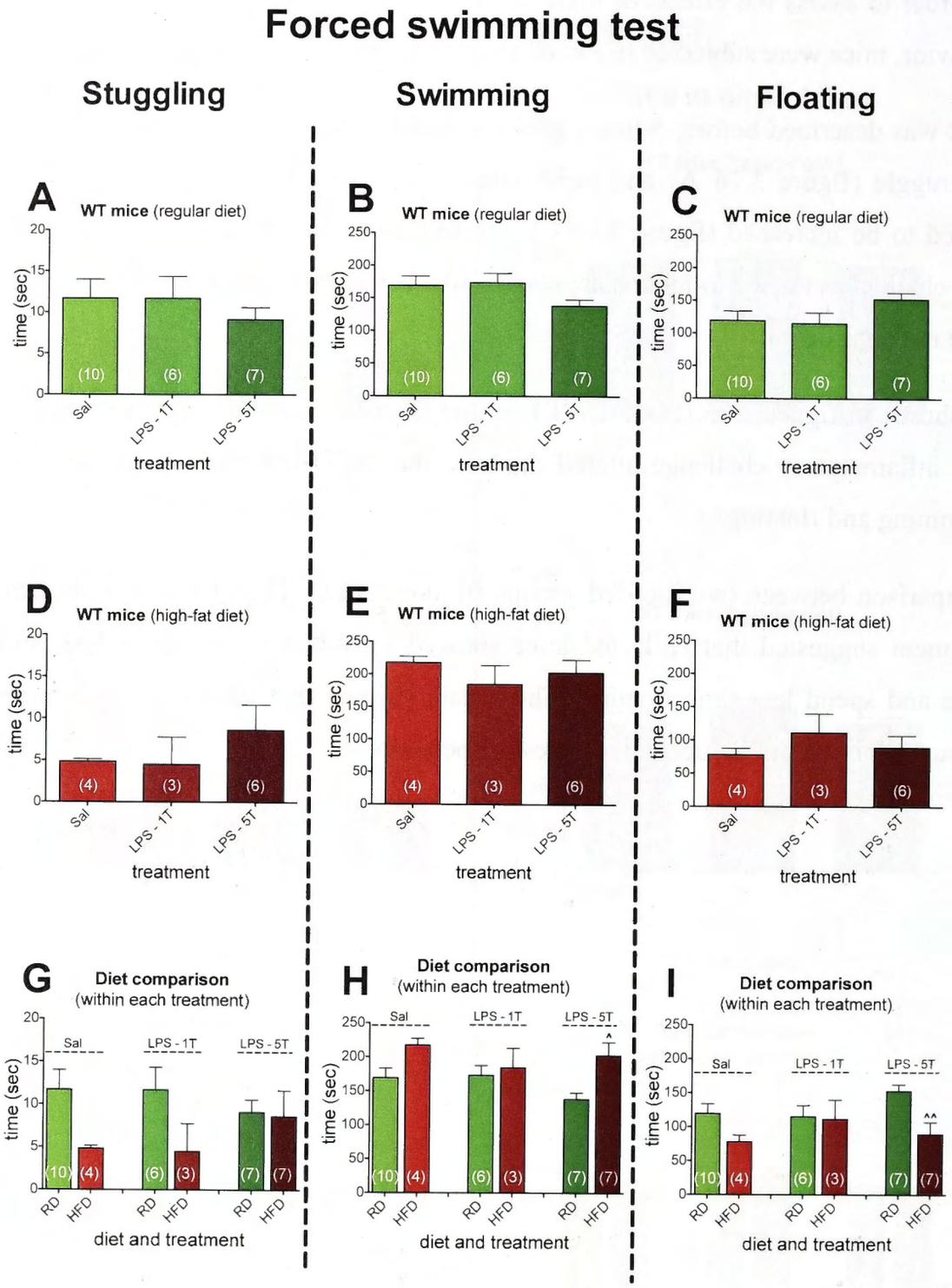


Figure 3.16: Effects of LPS inflammatory challenge and high-fat diet on depressive-like behavior in the forced swimming test: *Treatment comparison*: Time that A) RD and D) HFD-fed mice spent struggling; B) RD and E) HFD swimming and C) RD and F) HFD floating was measured in seconds and analyzed by one-way ANOVA (Tukey's multiple comparison test). *Diet comparison*: Time that mice spent G) struggling, H) swimming and I) floating was analyzed between RD and HFD-fed mice within the same treatment by unpaired T-test. Error bars represent standard errors. ^$p<0.05$, ^^$p<0.01$ vs. RD.

The novelty suppressed feeding test is used to ascertain anxiety-like behavior in rodents. Mice are food-deprived for 16 hours and then exposed to a new environment with a food pallet placed in the centre of arena. Since mice are hungry but also afraid

of open areas, they have to overcome their fear to approach the open area in order to eat. The faster they do it, the less anxious they are. This anxiogenic environment tests the fear to approach the food pallet in the centre of arena in order to feed. Thus, latency to eat is the main measure taken to assess the anxiety. It represents the total time the animal spent in arena until the moment the mouse took the first bite of food pallet. Afterwards the mice are transported to their home cage and total food intake is measured for a certain period of time (5 minutes). The home cage is a familiar environment and therefore this second part of the experiment is used to determine mouse food intake in a non-stressful environment.

As it may be observed in figure 3.17 A and C, latency to eat was not affected by single and repeated LPS injections, suggesting that LPS challenge did not elicit anxiety-like behavior in RD and HFD-fed mice.

Similarly, food intake in the home cage was not affected by single and repeated LPS injections in RD and HFD-fed mice (figure 3.17 B and D).

Comparison between RD and HFD-fed mice within each treatment revealed that the latency to eat of the HFD group was significantly higher suggesting that these mice were anxious to approach to the food pallet (figure 3.17 E).

The measurements of final food intake did not reveal significant difference between the RD and HFD groups. The food intake is the parameter that is used as verification that HFD-fed mice were in fact hungry, but were too anxious to approach to the food in open arena (figure 3.17 F).

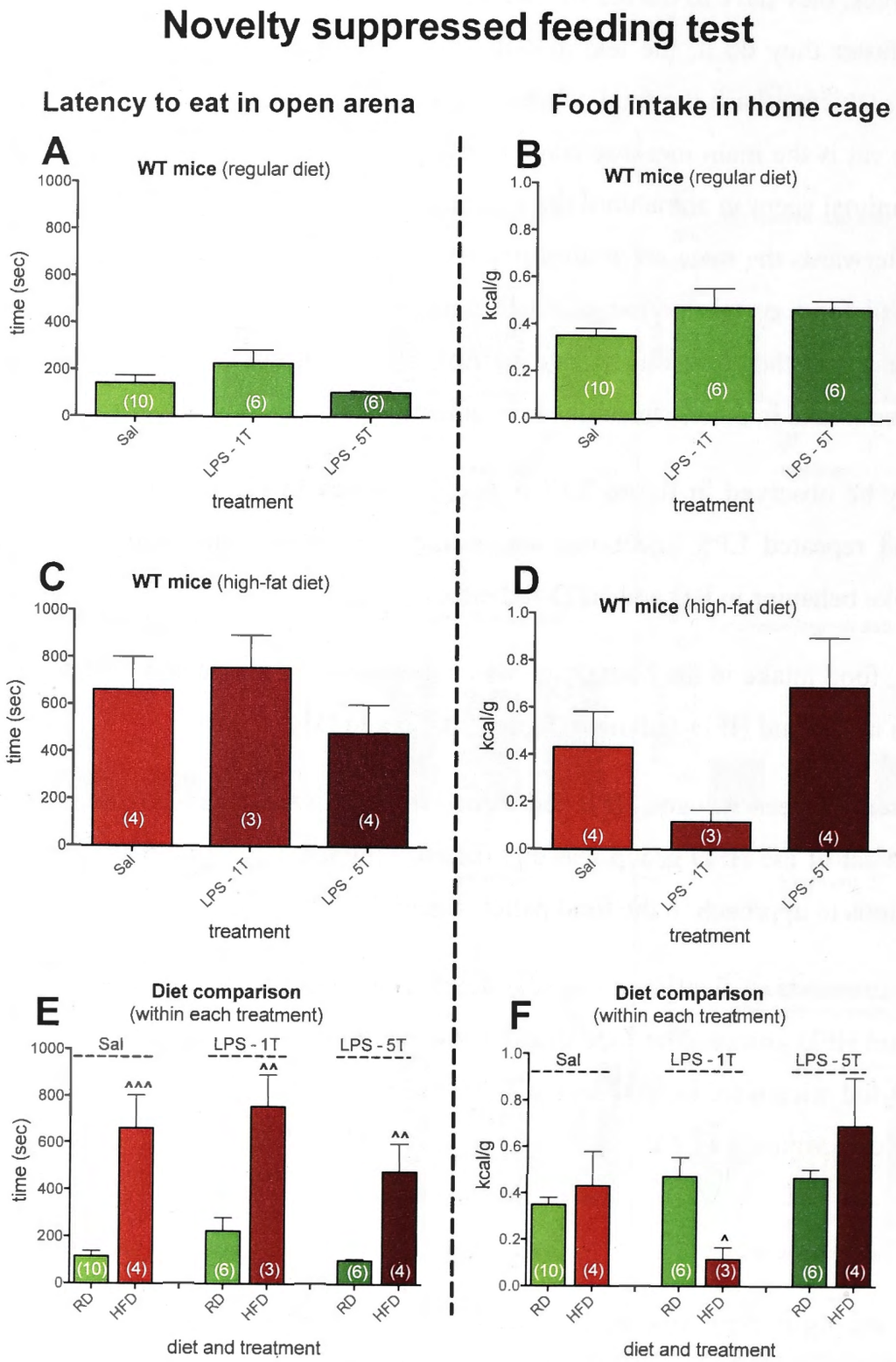


Figure 3.17: Effects of high-fat diet and LPS inflammatory challenge on anxiety-like behavior in the novelty suppressed feeding test: After sixteen hours fasting, mice were individually placed in a novel arena. *Treatment comparison*: Latency of A) RD and C) HFD-fed mice to take first bite of food pallet was measured in seconds. Food intake of B) RD and D) HFD-fed mice was measured in kcal/g. Data were analyzed by one-way ANOVA followed by Tukey`s post-hoc test. *Diet comparison*: E) Latency to eat and F) food consumption were compared between two diet-fed groups and within each treatment by unpaired T-test. Error bars represent the standard errors. ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs. RD.

3.4.3 Evaluation of memory and cognition

The prepulse inhibition test was used as an assessment of pre-attentive functioning in rodents [465, 466]. Evaluation of cognitive performance in this test is based on the animal`s ability to focus its attention on most silent information and exclude the disturbing effects of loud noise in the background [467]. Animal inability to canalize the information properly from the surrounding noise is thought to be related to over-activation of sensorimotor gating and cognitive defragmentation [468]. The prepulse inhibition test is an auditory test and its methodology is based on the principle that weak stimulus above background noise will reduce the magnitude of startle response in rodents and humans when it is followed with subsequent stronger sound stimulus. In this test a prepulse of 4, 8 and 16 dB above the background noise of 72 dB is presented prior to exposure to the stronger stimulus of 120 dB. Exposure to the prepulse stimuli prior to the stronger stimulus reduces the magnitude of the startle response. Therefore, it is expected that the percentage of inhibition of the startle response will be higher when the intensity of prepulse stimulus increases [469]. Previous studies have shown the relation between dopamine and its receptors in modulation of prepulse inhibition in rodents [381]. In particular, there is evidence suggesting that D1 and D2 receptors may be involved in regulation of prepulse inhibition [381]. Stimulation of either D1 or D2 receptors by their specific pharmacological agonist led to a reduction of prepulse inhibition in mice [470]. The percentage of startle response inhibition in the prepulse inhibition test has been related with the level of dopamine, as the dysregulation in DAT in mice led to an "overflow" of dopamine, which was associated with deficit in prepulse inhibition [380].

Mice of three genotypes (WT, Casp-1-/- and IL-1ra-/-) were exposed to prepulse stimulus of 4, 8 and 16 dB above background noise of 72 dB (i.e. 76, 80 and 88 dB). In the figure 3.18 (A, B and C) is presented the percentage of inhibition of startle response of three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) in order to assess the effect of single and repeated LPS inflammatory challenges on cognitive function within each genotype. Data collected from prepulse inhibition test have indicated that single and repeated LPS inflammatory challenges did not decrease or additionally exacerbate the attentive function in all three genotypes (figure 3.18 A, B and C).

In figure 3.18 (D, E and F) the percentages of inhibition of the startle responses among three different genotypes (WT, Casp-1-/- and IL-1ra-/-), within the same treated group, are presented in order to assess the role of IL-1 pathway in sensorimotor gating. Based on the results shown in figure 3.18 (D, E and F), the percentages of inhibition of the startle response in IL-1ra-/- mice were lower, but not significantly, than those observed in the control group (WT mice). Similarly, startle response inhibition in Casp-1-/- mice tended to be lower than in WT mice and reached significance in the saline-treated group (figure 3.18 D).

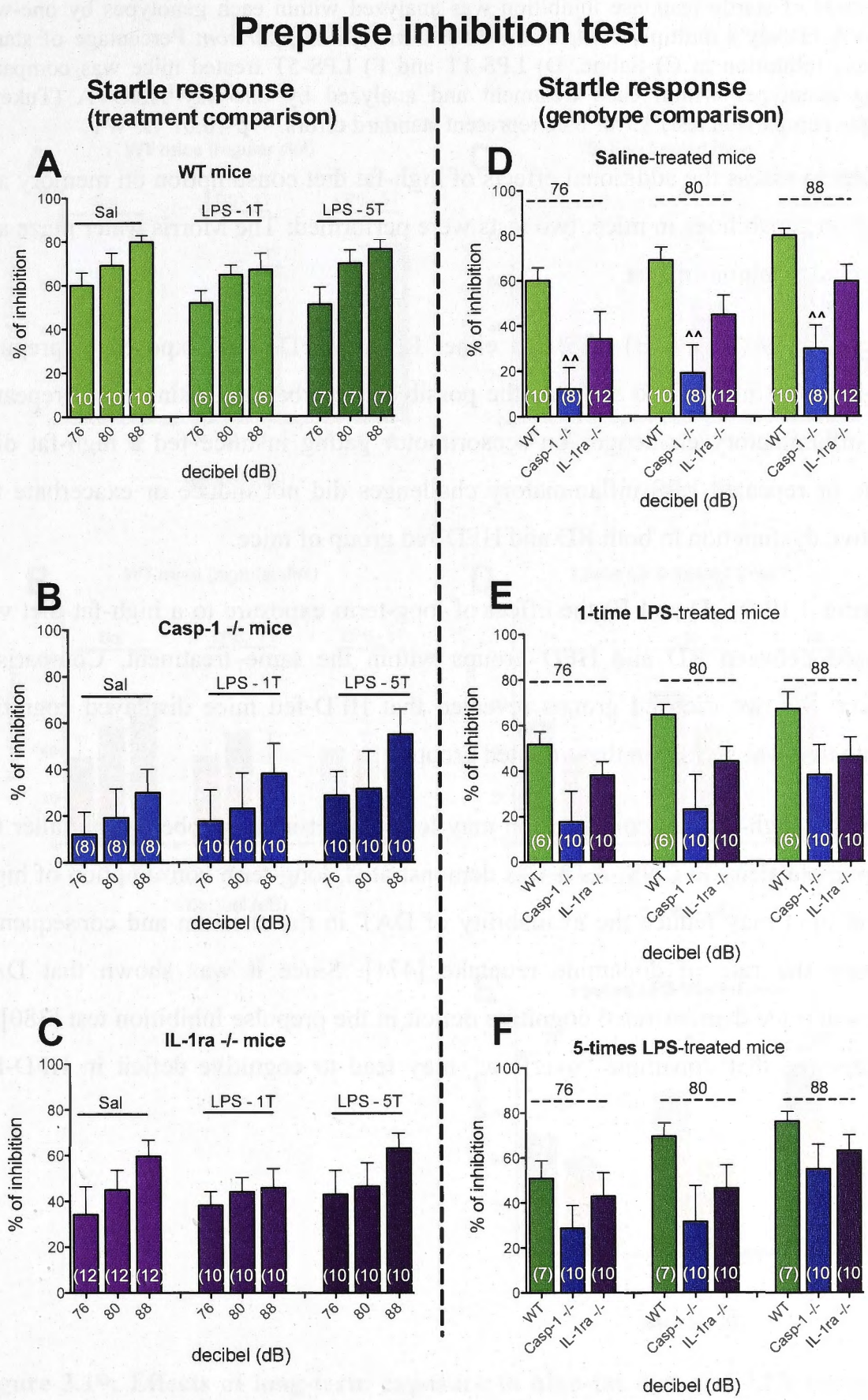


Figure 3.18: Effect of LPS inflammatory challenge on startle response in the prepulse inhibition test: Mice were placed in prepulse inhibition apparatus and exposed to the pulse alone (120 dB) and prepulse stimuli that were 4, 8 and 16 dB above the background noise of 72 dB, as described in chapter 2. *Treatment comparison*: Effect of single and repeated LPS injections in A) WT, B) Casp-1-/- and C) IL-1ra-/- mice on

magnitude of startle response inhibition was analyzed within each genotypes by one-way ANOVA (Tukey`s multiple comparison test). *Genotype comparison*: Percentage of startle response inhibition in C) Saline, D) LPS-1T and F) LPS-5T treated mice was compared among genotypes within each treatment and analyzed by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors. ^^$p < 0.01$ vs. WT.

In order to assess the additional effects of high-fat diet consumption on memory and cognition capabilities in mice, two tests were performed: The Morris water maze and the prepulse inhibition test.

In figure 3.19 (A and B) mice fed either RD or HFD were exposed to prepulse inhibition test in order to evaluate the possible exacerbation of single and repeated LPS inflammatory challenges on sensorimotor gating in mice fed a high-fat diet. Single or repeated LPS inflammatory challenges did not induce or exacerbate the attentive dysfunction in both RD and HFD-fed group of mice.

In figure 3.19 (C, D and E) the effect of long-term exposure to a high-fat diet was assessed between RD and HFD groups within the same treatment. Comparison between the two diet-fed groups revealed that HFD-fed mice displayed cognitive decline that was similar in three treated groups.

Prolonged high-fat diet consumption may lead to diet-induced obesity and alter the synaptic plasticity in CNS. As it was demonstrated, long-term consumption of high-calorie food may reduce the availability of DAT in the striatum and consequently decrease the rate of dopamine reuptake [471]. Since it was shown that DAT knockout mice demonstrated cognitive deficit in the prepulse inhibition test [380], it is suggested that dopamine "overflow" may lead to cognitive deficit in HFD-fed mice.

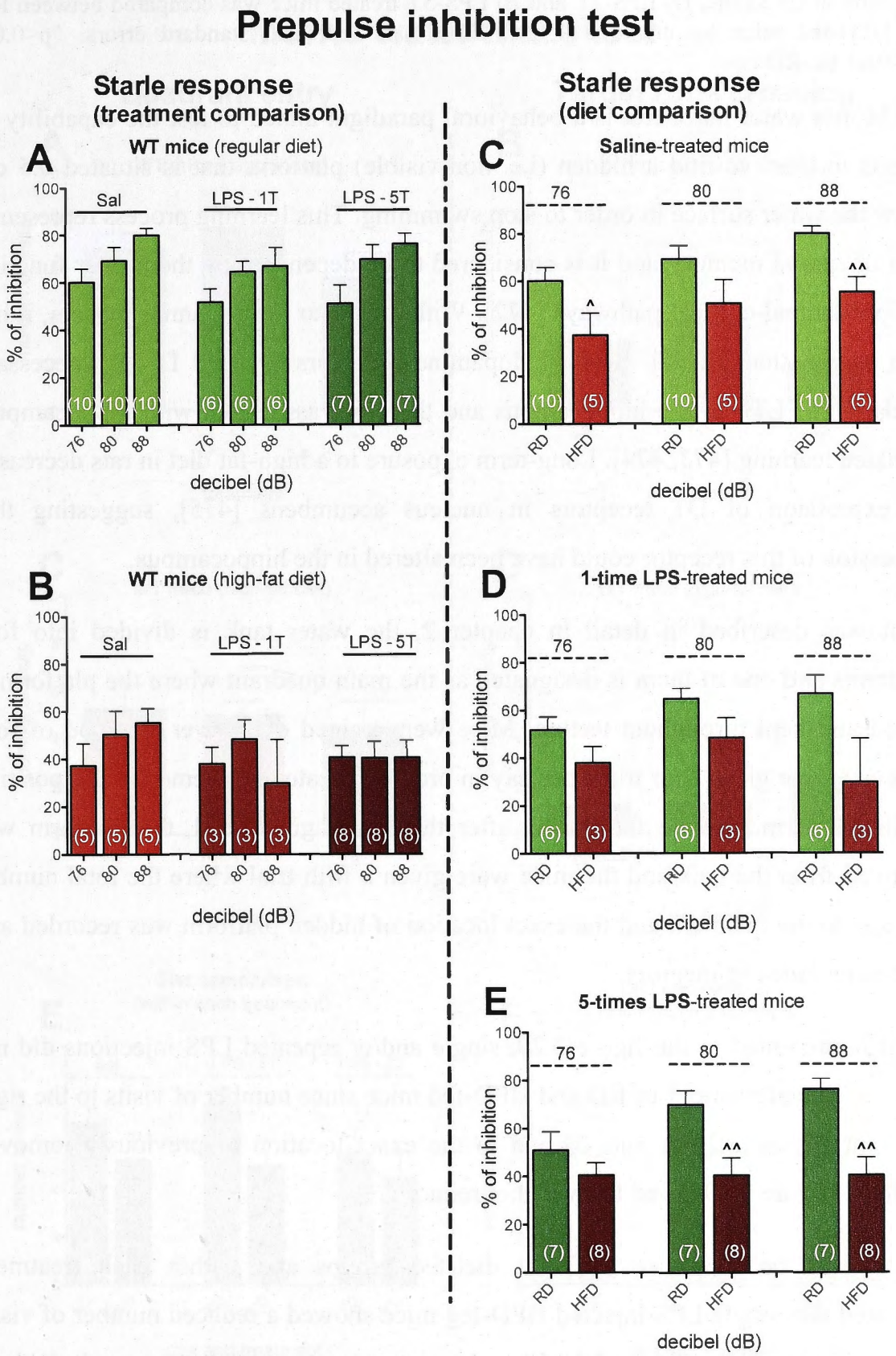


Figure 3.19: Effects of long-term exposure to high-fat diet and LPS treatment on startle response in the prepulse inhibition test: Mice were placed in prepulse inhibition apparatus and exposed to the pulse alone (120 dB) and prepulse stimuli that were 4, 8 and 16 dB above the background noise of 72 dB, as described in chapter 2. *Treatment comparison*: Effect of single and repeated LPS injections in A) RD and B) HFD-fed mice on magnitude of startle response inhibition was analyzed within each diet by one-way ANOVA (Tukey`s multiple comparison test). *Diet comparison*: Percentage of startle response

inhibition in C) Saline, D) LPS-1T and E) LPS-5T treated mice was compared between RD and HFD-fed mice by unpaired T-test. Error bars represent standard errors. ^$p<0.05$, ^^$p<0.01$ vs. RD.

The Morris water maze test is a behavioral paradigm aimed to test the capability of rodents to learn to find a hidden (i.e. non-visible) platform that is situated 0.5 cm below the water surface in order to stop swimming. This learning process represent a form of spatial memory and it is considered to be dependent on the proper function of hippocampal-cortical pathways [472]. With regard to the dopamine system, it has been shown that the D1 class of dopamine receptors D1 and D5 are necessary regulators of LTP in the hippocampus and therefore associated with hippocampus-mediated learning [473, 474]. Long-term exposure to a high-fat diet in rats decreased the expression of D1 receptors in nucleus accumbens [475], suggesting that expression of this receptor could have been altered in the hippocampus.

As it was described in detail in chapter 2, the water tank is divided into four quadrants and one of them is designated as the main quadrant where the platform is placed and kept throughout testing. Mice were trained daily over a period of four days and were given four trials per day in order to locate and memorize the position of the platform. On the fourth day after the fourth given trial, the platform was removed from the tank and the mice were given a fifth trial where the total number of visits to the quadrant and the exact location of hidden platform was recorded and used as an index of memory.

As it is presented in the figure 3.20, single and/or repeated LPS injections did not alter the spatial memory of RD and HFD-fed mice since number of visits to the right quadrant (figure 3.20 A and C) and to the exact location of previously removed platform (figure 3.20 B and D) were not reduced.

Comparison between two different diet-fed groups and within each treatment indicated that single LPS-injected HFD-fed mice showed a reduced number of visits to the right quadrant (figure 3.20 E) and to exact location of the removed platform (figure 3.20 F). These data suggest that there was a tendency for HFD-fed mice to display spatial memory impairment.

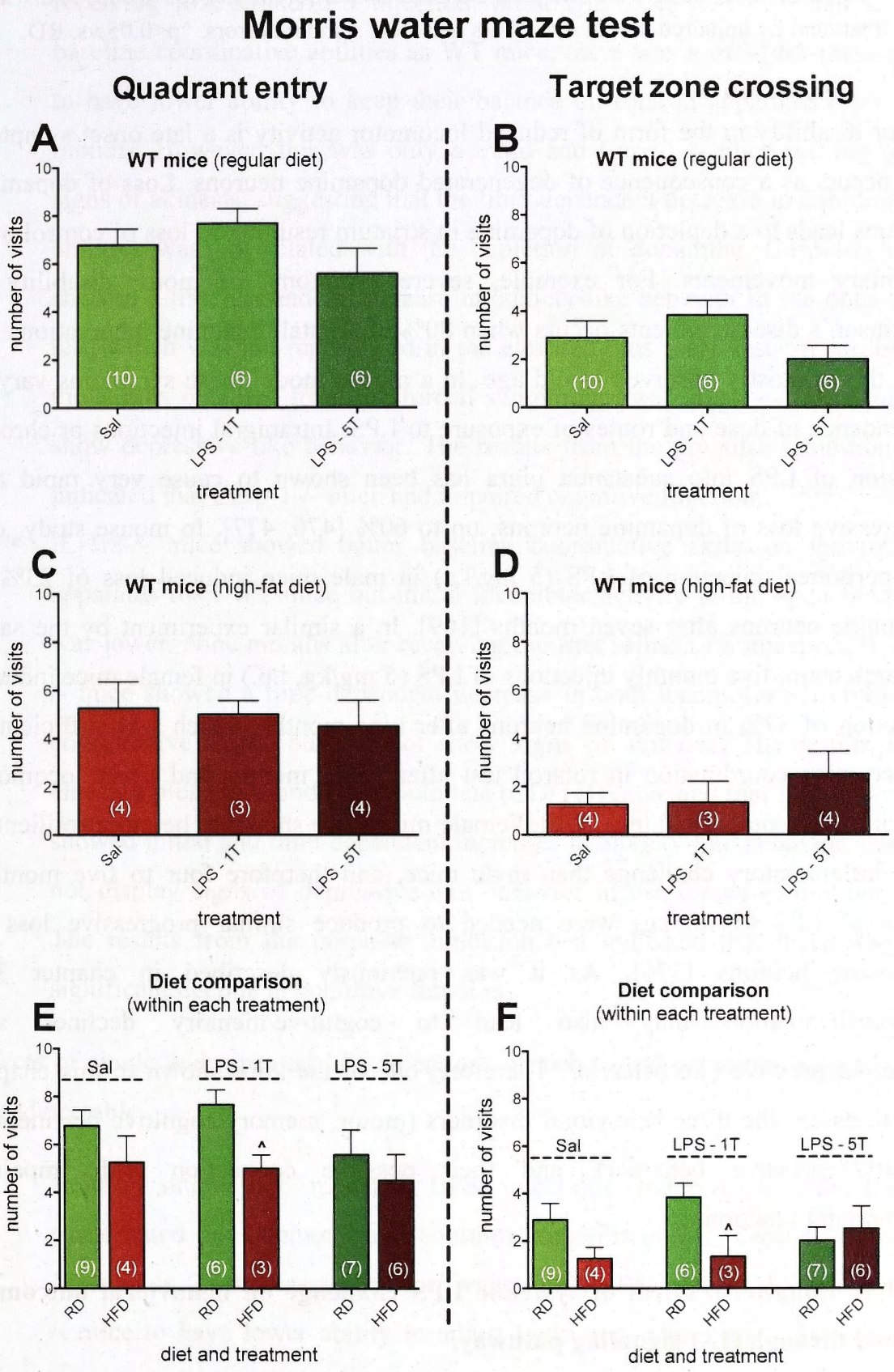


Figure 3.20: Effects of high-fat diet and LPS inflammatory challenge on memory performance in the Morris water maze test: After four days of training mice`s ability to memorize the right location of hidden platform was tested in Morris water maze test. *Treatment comparison*: Number of quadrant entry of A) RD and C) HFD-fed mice and target zone crossing of B) RD and D) HFD-fed mice were analyzed by one-way ANOVA (Tukey`s multiple comparison test). *Diet comparison*: E) Number of quadrant entry

and F) target zone crossing were analyzed between two different diet-fed groups and within each treatment by unpaired T-test. Error bars represent standard errors. ^p<0.05 vs. RD.

3.5 Discussion

Motor disability in the form of reduced locomotor activity is a late onset symptom that occurs as a consequence of degenerated dopamine neurons. Loss of dopamine neurons leads to a depletion of dopamine in striatum resulting in loss of control over voluntary movements. For example, severe symptoms of motor disability in Parkinson`s disease patients occurs when 80% of striatal dopamine innervations are lost; this is mostly observed in old age. In a mouse model these symptoms vary in dependence of dose and routes of exposure to LPS. Intranigral injections or chronic infusion of LPS into substantia nigra has been shown to cause very rapid and progressive loss of dopamine neurons, up to 60% [476, 477]. In mouse study, one intraperitoneal injection of LPS (5 mg/kg) in male mice induced loss of 23% of dopamine neurons after seven months [109]. In a similar experiment by the same research team, five monthly injections of LPS (5 mg/kg, i.p.) in female mice induced depletion of 37% in dopamine neurons after nine months, which was sufficient to reduce mice coordination in rotarod test after seven months and lately locomotor activity in the open-field test [376]. Female mice were shown to be more resilient to LPS inflammatory challenge than male mice, and therefore four to five monthly doses of LPS (5 mg/kg) were needed to produce similar progressive loss of dopamine neurons [376]. As it was previously described in chapter 3.1, neuroinflammation may also lead to cognitive/memory declines and anxiety/depressive-like behavior. Therefore, one of the aims shown in this chapter was to assess the three behavioral disorders (motor, memory/cognitive decline and anxiety/depressive behavior), and their possible connection with impaired nigrostriatal function.

AIM 1: Long-term effect of systemic LPS challenge on behavioral outcomes, exerted through IL-1 signaling pathway.

Major differences in behavioral outcomes were observed among the three genotypes (WT, Casp-1-/- and IL-1ra-/-):

- Casp-1-/- mice showed increased baseline locomotor activity in the open-field test and these mice remained hyperactive after nine months from

receiving first saline/LPS injection. Although Casp-1-/- mice had similar baseline coordinative abilities as WT mice, there was a trend for these mice to have lower ability to keep their balance on rotarod apparatus after nine months. However, this was only a trend and Casp-1-/- mice did not show signs of akinesia, suggesting that the time-dependent decrease in coordinative abilities was not related with the depletion of dopamine. Casp-1-/- mice showed a time-dependent increase in anxiety-like behavior in the open-field test, which was not reproduced in the elevated plus maze test. According to the results obtained from the forced swimming test, Casp-1-/- mice did not show depressive-like behavior. The results from the prepulse inhibition test indicated that Casp-1-/- mice had impaired cognitive function.

- IL-1ra-/- mice showed better baseline coordinative skills on the rotarod apparatus than WT mice but initial locomotor activity in the open-field test was lower. Nine months after receiving the first saline/LPS injection, IL-1ra-/- mice showed a time-dependent decrease in both locomotor activities and coordinative skills, but did not show signs of akinesia. The results from elevated plus maze and open-field test (CD/TD) indicated that IL-1ra-/- mice showed initial and time-dependent increases in anxiety-like behavior, but did not display signs of depressive-like behavior in the forced swimming test. The results from the prepulse inhibition test indicated that there was not significant decline in cognitive function.

Effects of single and repeated LPS injections on behavioral outcomes were modest or undetectable.

- Neither single nor repeated LPS injections induced or significantly exacerbated the locomotor and coordinative skills in WT, Casp-1-/- and IL-1ra-/- mice. There was a trend for five monthly LPS-injected WT and IL-1ra-/- mice to have lower ability to adjust their steps (forelimb akinesia). LPS treatment did not induce or exacerbate anxiety-like behavior of the three genotypes either in the open-field test (CD/TD) or in the elevated plus maze test. Five monthly LPS injections tended to induce depressive-like behavior in WT mice, whereas LPS effect was not observed in Casp-1-/- and IL-1-ra -/- mice. Results obtained from the prepulse inhibition test showed that single

and repeated LPS injections did not induce or exacerbate the cognitive impairment in the three genotypes.

1) Long-term effect of single and repeated LPS inflammatory challenges on locomotor abilities in mice.

Mice of three different genotypes (WT, Casp-1-/- and IL-1ra-/-) were used to evaluate the role of IL-1 on locomotor activity. Initial exploratory activities were significantly different among the three genotypes in the open-field test. Casp-1-/- mice had higher locomotor activity in open-field arena and conversely, IL-1ra-/- mice were the least active group. These observations are in the agreement with the previous study where systemic IL-1β administration in mice reduced their locomotor activities [478]. In contrast, mice lacking IL-1R1-/- signalling displayed increased locomotor activities [479]. Collectively, these observations suggest that motor activities in IL-1ra-/- and Casp-1-/- mice are inversely related with IL-1β signalling pathway.

The effect of single and repeated LPS inflammatory challenges on locomotor activity in the open-field test was then monitored nine months after the first saline/LPS injection. Direct comparison within each genotype and treatment indicated that locomotor activity in IL-1ra-/- mice significantly decreased after nine months regardless of treatment, while activity in WT and Casp-1-/- mice remained relatively unchanged. Percentages of differences in locomotor activity calculated between 0 and 9 months revealed that single and repeated LPS injections did not reduce the motor performance in any of the three genotypes. As it was shown previously that five LPS injections in female WT mice reduced locomotor activity after nine months [376], similar results could not be observed with single or repeated LPS injections. In another similar experiment conducted in two and half months old rats, single systemic LPS (5 mg/kg, i.p.) injection reduced locomotor activity in the open-field arena after seven days, but further exacerbation in motor hypoactivity was not observed after ten months [403]. The comparison among the three different genotypes and within the same treatment indicated that the decrease in locomotor activity in IL-1ra-/- mice was higher than in WT and Casp-1-/- mice. Long-term reduction in locomotor activity of IL-1ra-/- mice could be attributed to the excess of IL-1β signaling in the brain, as it was shown that in rats with chronic over-

expression of IL-1β by the adenoviral-mediated gene in the brain, open-field activity decreased [480]. Overall, it might be suggested that prolonged central rather than peripheral activation of IL-1β signaling may produce more significant decline in a motor activities.

Motor coordination was further evaluated in the rotarod test. Baseline assessment of coordination of all three genotypes on the rotating drum showed that IL-1ra-/- mice had better coordinative skills than WT mice. Evaluation of rotarod performance over a period of nine months indicated that coordination of all three genotypes decreased and it was not further exacerbated by the LPS treatment. Age related decline in rotarod performance in WT (saline) control group has been observed in other long-term studies even after eight months [481]. Comparison among the three genotypes and within same treatment showed that decrease in motor balance was pronounced in IL-1ra-/- mice in comparison to WT mice. Since chronic over-expression of IL-1β by the adenoviral-mediated gene in rat brains decreased locomotor activity in the open-field test [480], similarly it could be assumed that central overstimulation of IL-1β signaling in IL-1ra-/- mice led to decreased coordination.

Forelimb akinesia (loss or impairment of voluntary movements) is one of the parameters used to assess dysregulation of the dopamine system. It has been demonstrated that intranigral injection of LPS in rats produced forelimb akinesia [482]. In that regard, single and repeated LPS injections tended to reduce the number of adjusted steps in WT mice. Non-significant decrease has been observed in five LPS-injected IL-1ra-/- mice, while single and repeated LPS injections did not alter ability of Casp-1-/- mice to adjust steps. Comparison among genotypes revealed that the ability of Casp-1-/- and IL-1ra-/- mice was significantly better than in WT mice, which could have been due to the strain differences. Collectively, data suggest that repeated LPS injections tend to induce decline over voluntary movements in WT and IL-1ra-/- mice, which could be related to IL-1β signaling pathways, since Casp-1-/- mice were protected against LPS inflammatory challenge.

Overall, the effect of single and repeated LPS injections on locomotor impairment in all three genotypes was modest or undetectable. Although it was previously shown that five LPS injections (i.p.) induced reduction in locomotor and coordinative behavior in female mice over a period of 7 to 9 months [376], in the current study

neither single nor repeated LPS injections produced similar results in male mice. Significant time-dependent reduction of the locomotor ability and coordinative skills in IL-1ra-/- mice could have been attributed to a decreased level of dopamine and/or dopaminergic neurons.

2) Long-term effect of single and repeated LPS inflammatory challenges in anxiety/depressive-like behavior.

One of the parameters used in the assessment of anxiety-like behavior in mice is the ratio of central to total distance travelled in open-field arena [483]. Mice are by nature afraid of open areas and mostly prefer darker and enclosed environments. Therefore, the central area of the open-field arena may evoke anxiogenic behavior in rodents. Behavioral evaluation of the three genotypes at the baseline showed that IL-1ra-/- mice were prominently anxious in comparison to WT and Casp-1-/- mice. This anxiogenic behavior in IL-1ra-/- mice could have been related to the over-activated IL-1 signaling as it was shown that i.c.v administration of IL-1β induces anxiety-like behavior [483]. In a time frame of 0 to 9 months both Casp-1-/- and IL-1ra-/- mice became significantly more anxious and this behavior was not exacerbated by single or repeated LPS treatments. In regard to a caspase-1 signaling, inhibition of caspase-1 activity by the appropriate inhibitor (z-YVAD-FMK) has been shown to increase the NMDA receptor-mediated current in hippocampal slices *in vitro* [484, 485]. In connection with the previous statement, inhibition of NMDA signaling in dorsal hippocampus induced anxiolytic-like behavior in mice [486], suggesting that time-related increase in activity of NMDA signaling in Casp-1-/- mice could have resulted in anxiety-like behavior in the open-field test. On the other hand, WT mice did not show any signs of anxiety-like behavior after nine months, nor it was altered by single and repeated LPS injections.

Similar to the open-field test, the aim of the elevated plus maze paradigm is to evaluate the anxiety-like behavior in rodents. In this test mice`s innate fear for the open, unprotected and brightly illuminated arm of the maze is challenged against their desire to explore a new environment. Therefore, more anxious animals tend to spend more time in the closed arm. Acute LPS-induced synthesis and secretion of IL-1β has been suggested to be associated with the development of anxiety-like behavior in rodents [487]. In the present study, the long-term effect of single and

repeated LPS injections was not observed in any of the three genotypes. Comparison among genotypes indicated that IL-1ra-/- mice were the most anxious group of mice as they spent the least amount of time in the open-arm and consequently more time in the closed arm. On the other hand, Casp-1-/- mice seemed to display less anxious behavior, since these animals tended to spend more time in the open and consequently less time in closed arm in comparison to WT mice. The involvement of IL-1 in anxiety-like behavior is partially in agreement with previous studies where IL-1R1-/- deficient mice, were shown to display anxiolytic behavior in the elevated plus maze test [479].

The forced swimming test is used to assess depressive-like a behavior in rodents. The proportions of time that each animal spends in an immobile state (floating) or otherwise not struggling are used as indicators of depressive-like of behavior. Peripheral administration of LPS in mice has been shown to increase the duration of immobility in the forced swimming test [488]. The role of IL-1 in depression was demonstrated on an example of transgenic mice with deletion in IL-1R1, where chronic mild stress failed to induce depressive-like behavior in these mice [489]. Repeated LPS injections in WT mice seem to partially induce depressive-like behavior since there was a trend for these mice to struggle less and float more. In contrast, single and repeated LPS injections tended to even increase swimming and reduce floating in Casp-1-/- mice, suggesting that IL-1β-mediated inflammation was necessary to induce depressive-like behavior. These results are in agreement with recent published data in mice where it has been shown that the caspase-1 converting enzyme was necessary for the development of depressive-like of behavior following administration of LPS (i.c.v.) in mice [490]. Repeated LPS injections in IL-1ra-/- mice did not alter the time mice spent struggling or floating. Comparison among the three genotypes indicated that the only significant difference was that IL-1ra-/- mice spent significantly less time struggling than WT mice. Since the final outcome in IL-1ra-/- mice was not accompanied by increased immobility in the forced swimming test, it might be concluded that there was no significant difference among the three genotypes with regards to changes in depressive-like behavior.

Overall, observations suggest that IL-1ra-/- mice have shown baseline and time-dependent increases in anxiety-like behavior in open-field and elevated plus maze test that was not further exacerbated by single and repeated LPS injections. These

data imply that overstimulation of IL-1 might have contributed to anxiety-like behavior. In support of the previous statement, it was previously shown that i.c.v administration of IL-1β caused anxiety-like behavior in mice measured in the open-field and elevated plus maze test [483]. In particular, anxiety-like behavior caused by IL-1β was associated with abrogated sensitivity of cannabinoid CB1 receptors that control GABA synapses in the striatum [483]. On the other hand, time-dependent increases in anxiety-like behavior were observed in Casp-1-/- mice, despite tendencies to show anxiolytic behavior in elevated plus maze test. More studies are needed to clarify if Casp-1-/- mice become anxious in a time-dependent manner.

3) Long-term effect of single and repeated LPS inflammatory challenges on cognitive function.

It has been hypothesized that perturbations in the level of dopamine (excess or deficit) may impair cognitive function [491, 492]. The role of IL-1 signaling in attentive cognitive performance was assessed by prepulse inhibition test in three genotypes (WT, Casp-1-/- and IL-1ra-/-) of mice. Single and repeated LPS injections did not alter cognitive function in all three genotypes. It has been demonstrated that acute LPS injection in mice reduced prepulse inhibition startle response 24 hours post-injection [493]. Similarly, in another study acute LPS injections (i.p.) reduced the prepulse inhibition startle response in a dose-dependent manner [494]. It is plausible that the long-lasting effect of LPS on prepulse inhibition startle response was not longer present. Comparison among genotypes indicated that Casp-1-/- mice had lower startle response in comparison to WT mice and this was in particular significant in saline-treated group. As it was described earlier, inhibition of caspase-1 activity by the appropriate inhibitor (z-YVAD-FMK) increased the NMDA receptor-mediated current [484, 485]. Activation of NMDA receptors in primary hippocampal cultures isolated from fetal Wistar rats are shown to recruit more D1 receptors to the plasma membrane [495]. On the other hand, stimulation of D1 receptors by specific agonists disrupted the prepulse inhibition startle response in C57bl/6 mice [381]. Collectively, these observations suggest that possible over-activation of D1 receptors within the hippocampus of Casp-1-/- mice could have resulted in reduced inhibition of the prepulse inhibition startle response.

Data from the current study suggest that cognitive function in Casp-1-/- mice was partially affected by altered dopamine receptor signaling. On the other hand, no significant alterations have been induced by LPS inflammatory challenges. There was no similar significant impairment in cognitive function of IL-1ra-/- mice.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on behavioral outcomes.

Major differences in behavioral outcomes have been observed between RD and HFD-fed mice, whereas effects of single and repeated LPS injections were modest or undetectable:

- The long-term high-fat diet feeding in WT mice did not induce locomotor disabilities, nor was it exacerbated with single or repeated LPS injections. On the other hand, coordinative skills in HFD mice declined with time, but this decrease was not exacerbated by LPS treatments. HFD-fed mice showed signs of akinesia, bradykinesia and dyskinesia that were not exacerbated by single and/or multiple LPS injections. Despite the decline in coordinative abilities observed in rotarod test, HFD mice did not respond to the L-DOPA/carbidopa test. HFD-fed mice did not show anxiety-like behavior in open-field and elevated plus maze test, but they showed anxiety after overnight fasting in the novelty suppressed feeding test that was not exacerbated with LPS treatment. No effect of high-fat diet or LPS treatment on depressive-like behavior was observed in the forced swimming test. The results obtained from prepulse inhibition test indicated that overall saline and LPS-injected HFD mice showed cognitive impairment, and tended to have memory loss in the Morris water maze test.

1) Long-term effect of single and repeated LPS inflammatory challenges and high-fat diet consumption on locomotor abilities in mice.

Locomotor activity of mice fed with either regular or high-fat diet and subjected to treatments (Sal, LPS-1T and LPS-5T) was tested in the open-field test as an indication of possible motor dysfunction due to dysregulation of the nigrostriatal dopamine system.

Initial assessment of voluntary movements between HFD and RD-fed mice showed no difference. Similarly, nine months after the first saline/LPS injection, locomotor activity was not significantly changed within each treated group (Sal, LPS-1T and LPS-5T). The effect of single and repeated LPS injections within each diet-fed group (RD or HFD) was modest or undetectabl. On the other hand, it was previously described that locomotor impairment observed after nine months in female mice was induced with five monthly LPS (i.p.) injections [376]. Similar experiment conducted in male rats showed that single systemic LPS (5 mg/kg, i.p.) injection reduced locomotor activity in the open-field arena after seven days, but further exacerbation in motor hypoactivity was not observed after ten months [403]. Comparison between RD and HFD-fed mice and within same treatment indicated that there was no significant difference between locomotor activities of these mice. Therefore, these data suggest that high-fat diet and additional LPS treatment did not decrease motor activity.

Mice coordination has been tested in the rotarod test and as it was described previously, this test is able to detect an imbalance in the dopamine system [376]. The ability of RD or HFD-fed mice to keep their balance on the rotarod apparatus during baseline was not different between the two groups. Nine months after the first saline/LPS injection, the motor coordination in both RD and HFD mice was impaired in comparison to their individual baseline abilities. Reduced motor performance in RD mice, which was also observed in saline-treated mice is probably a consequence of the aging of the animal as it has been shown that coordination ability of C57bl/6 mice declines with the passage of time [496]. The evaluation of LPS effect within each diet-fed group showed that decline in rotarod performance was not further affected by single or repeated LPS injections. Even though previous studies have reported the first significant decline in the rotarod test after 7 months post-injection in five LPS-injected female mice [376], in the present studies similar results were not shown after nine months. The present results appear to be in agreement with the recent study in which locomotor disabilities were not exacerbated after 10 months after the first systemic LPS injection [403]. The direct comparison between RD and HFD-fed mice within each treatment revealed that reduced coordination in HFD-fed mice was significantly more pronounced. These observations may suggest that this significant decrease in the coordination of HFD-

fed mice may have resulted from a decline in the striatal dopamine level or loss of dopamine neurons. Although the HFD group was fed a fat enriched diet for a several months, these mice did not gain a significant amount of body weight (chapter 4). Thus, decreased rotarod performance in HFD mice was not due to increased body weight, which was described as one of the elements that might reduce the balance of mice on rotarod apparatus [497]. However, as it will be described in chapter 4, these mice were metabolically obese.

Consumption of a high-fat diet did not impair the muscle strength in mice as it was demonstrated with the grid test. Therefore, coordinative disabilities observed in HFD-fed mice could not be attributed to the possible impairment of neuromuscular function.

Twelve months after first saline/LPS injection mice were subjected to L-DOPA/carbidopa test with the aim of evaluating if the reduction in motor coordination in the rotarod could be attributed to the decrease of dopamine in the nigrostriatal circuit. Results obtained from RD-fed mice showed that single and 5-times LPS-injected mice positively responded to the L-DOPA/carbidopa treatment, which suggests that loss of dopamine overall might have been modest. On the other hand, reduced, but not significantly, motor abilities of single and repeated LPS-injected HFD-fed mice were not improved with L-DOPA/carbidopa treatment. One of the possible explanations is the fact that HFD-fed mice have slower clearance of dopamine from synaptic clefts due to the impaired function of DAT [471]. This suggests that HFD-fed mice had systemic "overflow" of dopamine and additional supplementation in the form of L-DOPA/carbidopa did not improve the motor impairment that was previously described in these mice.

The pole test, as described previously, is used in an assessment of bradykinesia (slowness of movement), which is also one of the symptoms related with nigrostriatal dysfunction [388]. Two parameters are used in this test: a) time to turn around and b) time to climb down the pole. Animals that show signs of bradykinesia need more time to turn around and climb down the pole. The single and repeated LPS injections slightly increased the time of RD and HFD-fed mice to turn around with no obvious effect on mice`s abilities to climb down the pole. Comparison between RD and HFD-fed mice showed that HFD-fed mice had the tendency to take

more time to turn around and significantly more time to climb down the pole. This data could be supported with the behavioral experiments performed on DAT -/- mice that were shown to display a motor deficit in the pole test, which was related to the hyperdopaminergic tone in dorsolateral neostriatum [498]. Therefore this statement might imply that reduced DAT function in HFD-fed mice could have resulted in symptoms of bradykinesia.

The stepping test was used to assess akinesia, symptom that is characterized by the impairment to initiate steps and it was related with the loss of positive TH-neurons [457]. Single and five monthly LPS injections tended to decrease the number of adjusted steps in RD-fed mice, whereas LPS treatment did not produce any decrease in HFD-fed mice. Comparison between RD and HFD-fed mice indicated that HFD-fed mice had a significantly fewer number of adjusted steps. For example, akinesia has been pharmacologicaly induced in mice by inhibition of D2 receptors [499]. Since high-fat diet feeding and obesity in mice was associated with decreased density of D2 receptors [500], lower availability of D2 receptors in HFD-fed mice could have induced symptoms of akinesia.

The hind limb clasping test has been used for the evaluation of dyskinesia (presence of involuntary movement and diminished voluntary movements). Mice were scored based on the severity of the condition as it was described in detail in chapter 2. Although single and repeated LPS injections did not impair the symptoms in RD and HFD-fed mice, the comparison between the two groups indicated that HFD-fed mice had shown significant neurological/motor impairment. Clasping behavior has been observed in DAT -/- mice [501] implying that the motor impairments observed in HFD-fed mice might be related with a reduced level of dopamine transporters.

Collectively, these data suggest that LPS treatment induced modest or undetectable effects in both RD and HFD-fed mice. Locomotor and coordinative impairments were more emphasized between RD and HFD-fed mice and might have been related with decreased striatal dopamine and/or impaired dopamine neurotransmission. Single and repeated LPS inflammatory challenge was not exacerbated by high-fat diet consumption.

2) Long-term effect of single and repeated LPS inflammatory challenges and high-fat diet consumption in anxiety/depressive-like behavior.

Anxiety-like behavior in RD and HFD-fed mice was evaluated in the open-field test. The ratio of central over total distance (CD/TD) was used as an indicator of animal anxiety towards the open illuminated environment. Baseline comparison between RD and HFD-fed mice suggests that there was no difference between these two groups. Nine months after the first saline/LPS injection, there was no significant decline within each treated group (Sal, LPS-1T and LPS-5T) of RD and HFD-fed mice. Similarly, no effects of single and repeated LPS injections within each diet-fed group were observed. Finally, direct comparison between RD and HFD-fed mice did not reveal any significant differences. Although, high-fat diet consumption in rodents has been linked to anxiety-like behavior [502], in the current test results did not indicate that HFD-fed mice suffered from increased anxiety-like behavior.

The elevated plus maze test was used to assess the effect of long-term high-fat diet consumption and LPS inflammatory challenge on anxiety-like behavior. It has been demonstrated that high-fat diet consumption induced anxiety-like behavior, which was observed by the reduced time that rats spent in the open arm of elevated plus maze [503]. There were tendencies of single and repeated LPS-injected mice, fed either a RD or HFD to spend less time in open arms of the maze, whereas no particular LPS effect was observed on time mice spent in closed arm of the maze. Comparison between RD and HFD mice suggested that high-fat diet did not induce anxiety-like a behavior in mice.

The third test used in the assessment of anxiety-like behavior in mice was novelty-suppressed feeding test. The role of the dopamine system in regulating anxiety has been previously described in chapter 3.1.2. Briefly, activation of glutamate releasing neurons in the limbic system by CRH leads to anxiety-like behavior, whereas release of dopamine in mid-brain induced by CRH evokes less fearful behavior [433]. As it was described in chapter 3.4.2, the novelty suppressed feeding test is a behavioral paradigm where a food-deprived animal has to overcome its natural fear of a novel environment and approach to food pallet in the centre of the arena in order to feed. The results obtained from the novelty suppressed feeding test indicated that LPS treatment did not produce or exacerbate anxiety-like behavior in RD and HFD-fed mice. The elapsed time that mice took to approach to the food pallet in centre of arena indicated that HFD-fed mice were significantly more anxious than RD mice. In order to understand if this delayed latency for HFD-fed mice to eat was due to the

anxiety and not due to the factor of being less hungry, mice were individually placed in their original home cages and the amount of consumed food was recorder over the next 5 minutes. The results obtained from both RD and HFD groups showed that mice from both groups consumed a similar amount of food, suggesting that HFD-fed mice were indeed more anxious than RD mice.

As it may be concluded, anxiety-like behavior in HFD-fed mice was only confirmed in the novelty suppressed feeding test, whereas analysis of the results obtained from open-field and elevated plus maze suggested that HFD-fed mice did not show anxiety-like behavior. The explanation for this non-consolidating data has been addressed with the possibility that 16 hours of fasting in HFD mice could have evoked heightened anxiety in these mice. It was observed that 24 hours of short-term fasting, which is considered as a physiological stressor, increased anxiety in HFD-fed mice, as their burrowing increased in comparison to when these mice were in a fed state [504]. Burrowing resembles digging or nesting in rodents and reflects depressive/anxiety-like behavior in rodents [505]. Similarly, short-term exposure to HFD increased anxiety in mice, as observed by increased burrowing behavior [502]. It has been demonstrated that 16 hours overnight fasting increased the plasma level of corticosterone by 2-fold in WT mice [506]. Moreover, it has been shown that six weeks of HFD consumption in mice resulted in increased anxiety-like behavior and HPA hypersensitivity to stress [507]. Thus there is a possibility that food deprivation increased the anxiety behavior in HFD-fed mice that could not have been detected in the previous two behavioral tests.

High-fat diet consumption has been shown to promote depressive-like behavior in rodents [508]. Depressive-like behavior was assessed in the forced-swimming test. There was a tendency for 5-times LPS-injected RD mice to struggle less and float more, suggesting that repeated LPS challenges partially induced depressive-like behavior. On the other hand, the effect of single and repeated LPS inflammatory challenges did not induce or exacerbate the depressive-like behavior in HFD-fed mice. The comparison between RD and HFD-fed mice showed that HFD-fed mice tended to struggle less, swim more and float less. These data suggest that HFD-fed mice did not show signs of depressive behavior.

Overall, data suggests that LPS treatment did not induce or exacerbate anxiety/depressive-like behavior in either RD or HFD-fed mice. The only significant effect of HFD that has been observed was in the anxiety-like behavior in novelty suppressed feeding test, which might have been driven by stress due to overnight fasting.

3) Long-term effect of single and repeated LPS inflammatory challenges and high-fat diet consumption on memory and cognition.

The possible role of LPS inflammatory challenge and high-fat diet consumption on cognitive impairment in mice was assessed with prepulse inhibition test. As it was described before in chapter 3.4.3 cognitive functions are processed by the hippocampus. Single and repeated LPS injections did not alter the cognitive functions in either RD or HFD-fed mice. The detailed comparison between the two groups, indicated that HFD-fed mice showed a certain degree of cognitive impairment in all three treated groups (Sal, LPS-1T and LPS-5T) based on lower inhibition of the startle response. As it was previously described in the chapter 3.4.3, the deletion of DAT in mice is related to the deficit in the prepulse inhibition test [380]. Since it has been demonstrated that chronic high-fat diet consumption reduced the availability of DAT in striatum [380], this suggests that reduced dopamine reuptake may lead to the cognitive deficit observed in HFD-fed mice [471].

The role of proinflammatory cytokines in hippocampal-dependent memory was described in an animal model [509-511]. Acute peripheral administration of LPS in rodents induced the expression of IL-1β in the brain, which was associated with spatial memory impairment in a water maze [509-511]. Memory impairment in the current study was assessed in the Morris water maze test. As it was described before, mice were trained during four days to allow them to memorize the position of the submerged platform (0.5 cm from water surface) in order to stop swimming and to escape from the water. On the forth day, the platform was removed from the water tank and the number of visits to the right quadrant and exact position (target crossing) of the platform were used as parameters for assessment of their memory capabilities. The effect of single and repeated LPS injections on memory functions was not observed in HFD-fed mice, while only modest decline in spatial memory was noticed in 5-times LPS-injected mice fed a RD. It has been demonstrated that

sustained expression of IL-1β in hippocampus of IL-1β (XAT) transgenic mice impaired spatial memory of mice in Morris water maze test [512], which suggests that hippocampal levels of IL-1β after repeated LPS injections were not sufficient to induce significant memory decline in the current study. On the other hand, there was tendency for the HFD-fed mice to have fewer visits to the quadrant and exact location of the removed platform. It is suggested that a high-fat diet induces chronic neuroinflammation in the brain that may constitute the link with memory impairment [446]. In particular, levels of proinflammatory cytokines such as TNF-α and IL-6 were found elevated in the cortex of HFD-fed mice [446]. Moreover, recent studies have associated the desensitization of protein kinase B (Akt) signaling pathway that is coupled to leptin receptor in the hippocampus of HFD-fed mice to spatial memory impairment [513], emphasizing the important role of leptin signaling integrity in the brain. Cognitive decline in HFD-fed mice was also attributed to the desensitization of NMDA receptors in the hippocampus and it was also observed that this process was compatible with the development of leptin resistance [514]. On the other hand, insulin resistance in HFD-fed mice decreased the release of dopamine and its clearance from synaptic clefts [12]. As it was reported that insulin resistance contributes to cognitive decline [448], this might explain a possible connection between dopamine signaling and insulin resistance-induced cognitive decline. In regards to the role of the dopamine system in memory function, it was previously described (chapter 3.4.3) that D1 receptors are associated with hippocampus-mediated learning. Long-term exposure to palatable food in rats reduced the expression of the D1 receptor in nucleus accumbens [475] suggesting that similar alteration in D1 expression could have occurred in the hippocampus.

These observations suggest that high-fat diet consumption partially induced memory impairment through the impairment in neurotransimision in the hippocampus, which could have been related with leptin resistance and possibly to down-regulation of D1 receptor expression. Cognitive impairments induced by long-term high-fat diet consumption could have been related with lower activity of DAT.

Conclusions:

AIM 1: Long-term effect of systemic LPS challenge on behavioral outcomes, exerted through the IL-1 pathway.

- The long-term effects of single and/or repeated LPS challenges modulating the behavior of the three mouse genotypes (WT, Casp-1-/- and IL-1ra-/-) were modest or non-detectable.

- Significant differences in behavioral outcomes were observed among the three genotypes. Genotype effect was observed in the following parameters:

- IL-1ra-/- mice showed a time-dependent reduction in locomotor activities in the open-field test and coordinative abilities in the rotarod test. Time-dependent increase in anxiety-like behavior was observed in the open-field and later in the elevated plus maze test. IL-1ra-/- mice did not display cognitive impairment.
- Casp-1-/- mice had preserved locomotor activity but showed tendencies towards decreased coordinative abilities in the rotarod test. Casp-1-/- mice demonstrated anxiety-like behavior in open-field test, but also tended to show anxiolytic behavior in the elevated plus maze test by the end of the *in vivo* study. Moreover, Casp-1-/- mice displayed altered cognitive function in the prepulse inhibition test.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on behavioral outcomes.

- Long-term high-fat diet consumption did not exacerbate LPS effect on behavioral outcomes.

- Significant differences in behavioral outcomes were observed between two diet-fed groups of mice. Diet effect was observed in the following parameters:

- High-fat diet-fed mice demonstrated impaired coordinative motor ability in the rotarod test, but not locomotor activities in the open-field test. HFD-fed mice showed symptoms of akinesia, bradykinesia and dyskinesia.
- HFD-fed mice showed sign of anxiety-like behavior in the novelty suppressed feeding test, possibly induced by overnight fasting.
- High-fed mice showed impairment in cognitive function and tended to have reduced memory function.

4. Effect of peripheral inflammatory challenge and high-fat diet on metabolic outcomes.

4.1 Introduction

Metabolic perturbations such as type-2 diabetes, insulin resistance, glucose intolerance and leptin resistance may alter the proper function of the dopamine system (chapter 1.11) and possibly be related to some of the behavioral outcomes that have been described in chapter 3.

As it was described before, single systemic LPS injection in male mice caused significant and delayed loss of dopamine neurons after seven months [376]. The intraperitoneal LPS injection induced activation of the innate immune system and concomitant release of proinflammatory cytokines such as IL-1 and TNF-α in the periphery and within the brain [3]. It has been shown that increased serum level of IL-1 induced transient diabetes in rats [515] and TNF-α has been linked with insulin resistance in obese mice [516].

According to WHO, the prevalence of obesity has doubled from 1980 to 2008 [318]. Obesity is considered as a state of low-grade chronic inflammation since food high in fat and sugar can act as an inflammatory component triggering the activation of TLR4 in innate immune cells [6]. With the correspondent activation of the innate immune system, it is proposed that there might be a connection between obesity induced metabolic disorders, inflammation and neurodegenerative diseases [7-9]. High-fat consumption is related with weight gain, which further contributes to the development of type-2 diabetes, glucose intolerance, leptin and insulin resistance as described in chapter 1.11. Thus, obesity can trigger chronic inflammatory and metabolic imbalances affecting dopaminergic neurotransmission.

For example, it has been shown that dopamine content was reduced in the nigrostriatal system of diabetic rats [10]. In human studies, diabetes has been shown to augment motor impairment in Parkinson`s disease patients [517]. Development of insulin resistance in high-fat diet-fed rats reduced dopamine release and its reuptake

in the synaptic vesicles [12]. In regards to leptin, leptin resistance may not influence the basal level of dopamine, but it can reduce the total capacity of dopamine cells to secret dopamine [11]. In fact, it has been shown that leptin regulates the expression of dopamine transporters [13].

Since obesity is considered as a low-grade chronic inflammatory state, the additional implementation of single and/or repeated LPS injections as peripheral inflammatory challenge was hypothesized to exacerbate metabolic disorders that are observed in obesity [6]. For example, the acute LPS inflammatory challenge augmented the IL-1β induced immune activity in the periphery and the brain in diabetic *db/db* (leptin receptor deficient) mice, which was associated with a loss of IL-1β counter-regulation [518].

In the current study, several metabolic outcomes were assessed to understand the possible impact of high-fat diet induced obesity in combination with long-term effects of LPS injections. Therefore, glucose tolerance, insulin and leptin levels were measured as possible factors affecting the dopamine system. Before an assessment of these biochemical markers, animal food intake, body weight and body length were monitored over time in order to better understand the effect of LPS and high-fat diet consumption on the development of metabolic disorders.

4.1.1 Control of food intake by CNS and the role of leptin in feeding control

Food intake is a process controlled by the CNS and it is stimulated by sensations such as hunger, craving, pleasure and sense of reward [519]. The hypothalamus is the main brain region responsible for food intake [520]. An essential hypothalamic control over the feeding process through integration of nutritional information is established by neuropeptides that are secreted from two neuronal populations situated in the arcuate nucleus [521, 522]. One neuronal population secretes neuropeptides that stimulate the appetite, also known as orexigenic peptides: neuropeptide Y (NPY) and agouti-related protein (AGRP) [521, 523], whereas the other one secrets anorexigenic peptides such as pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcripts (CART) [521, 523]. The neurons that secret orexigenic and anorexigenic peptides predominantly project their axons to other neurons located in PVN, lateral hypothalamic area (LHA), perifornical area (PFA), ventromedial and dorsomedial nuclei [519, 524].

Blood glucose level was thought to be a key initiator of feeding behavior [525] until it was discovered that other factors released from adipose tissue could signal the brain and control food intake [526]. In that sense, leptin [328] and insulin [527] were recognized as essential factors in controlling body weight and feeding behavior, whereas the communication between the gut and the brain has been considered to be mostly involved in control of satiety, regardless of metabolic requirement. The key role of leptin is carried out via its receptors (ObRb) expressed in the hypothalamus [523]. Leptin receptors are primarily expressed in several regions of the hypothalamus, such as arcuate nucleus, PVN, the dorsomedial nucleus and lateral hypothalamic area [523]. During fasting, leptin decreases the activity of NPY and AGPR neurons and the secretion of the orexigenic peptides (NPY and AGRP) [528, 529]. Orexigenic neurons project to PVN and stimulate food intake and decrease of energy expenditure, contributing in that manner to weight gain [519]. In a fed state, an increase in leptin level stimulates secretion of anorexigenic peptides such as α-melanocyte-stimulating hormone (α-MSH) and CART from arcuate nucleus that further project to LHA and PFA, which results in reduction of food intake and weight loss [529, 530]. Leptin also inhibits appetite through activation of dopamine and GABA neurons in the mesolimbic pathway [531].

In human and animals studies it was shown that under pathological conditions induced by activation of the innate immune system, food intake is disturbed as appetite decreases [532, 533]. Systemic inflammation causes disruption of neuronal circuits between periphery and the brain resulting in an imbalance of homeostasis of food intake and energy expenditure, which collectively leads to the development of anorexia and weight loss [534]. This pathological condition of anorexia is caused not only by bacterial products but may also be caused by high levels of cytokines [535]. Mice deficient in TLR4 exposed to LPS were shown to be resistant to anorexia [536]. Similarly, IL-1R1-/- mice showed resistance to weight loss after being challenged with IL-1β [537]. Activation of both TLR4 and IL-1R1 elicit inflammatory reactions via common signaling pathways, which involves recruitment of MyD88 [538]. In MyD88-deficient animals, LPS-induced synthesis of cytokines in the brain was significantly attenuated, which further prevented anorexia [538].

The effect of a high-fat diet on increased food intake is partially regulated via obesity-induced diabetes. Hyperphagia (a state of increased food intake) is tightly regulated by the hypothalamic NPY [539]. Since obesity is linked to the development of insulin resistance and hyperinsuilnemia it has been demonstrated that acute hyperinsuilnemia, stimulates the secretion of NPY, which in turn leads to increased appetite and food intake [540]. In particular, it is believed that diabetes in rats may alter the ability to utilize fat and carbohydrates from food [541]. Therefore, as a consequence of alteration in fuel oxidation, it is thought that diabetes may indirectly influence the expression of NPY that in turn would lead to increased food intake.

For all of the above, the putative effect of LPS inflammatory challenge and high-fat diet-induced obesity on food intake was assessed and will be discussed in chapter 4.4.1.

4.1.2 Effect of inflammation and high-fat diet consumption on body weight gain

In order to maintain the homeostatic balance, food intake, energy expenditure and storage of excess energy in the form of fat reserves has to be regulated by the actions of CNS. The CNS communicates with peripheral organs sensing the status of energy reserves and balancing it with the control of food intake and energy expenditure. As it was described before, leptin controls weight gain by suppressing appetite [329]. Its plasma levels correlates with fat mass and body weight. Thus, leptin levels rise with increased body weight or fall during weight loss [542].

Several inflammatory markers such as IL-1 and TNF-α were shown to stimulate leptin release [543]. It has been found that TNF-α stimulates leptin production through interaction with its receptor on adipocytes [544]. Likewise, acute exposure to LPS in fasting animals resulted in elevated levels of circulating leptin to almost the same extent as it was found in fed animals [545]. Overall, increased leptin levels during inflammation reduce food intake and lead to weight loss [546].

Consumption of a high-fat diet positively correlates with accumulation of adipose tissue [547]. The extent of weight gain is partially influenced by the irregular oxidative rate of ingested fat-enriched food [548]. On the other hand, continued feeding on a high-fat diet induces central leptin resistance accompanied with weight gain [549].

The effect of single and repeated LPS inflammatory challenge and high-fat diet food intake on body weight gain will be discussed in the section 4.4.2.

4.1.3 Effect of inflammation and high-fat diet on longitudinal body growth

The bone longitudinal growth and muscle development are dependent on growth hormone (GH), which is secreted from the pituitary gland located in *sella turcica* base of the brain. The secretion of GH is stimulated by the growth hormone-releasing hormone (GHRH), which is released from neurosecretory nuclei of the hypothalamus [550]. The control over GH secretion is also modulated by its inhibitor namely, growth hormone-inhibiting hormone (GHIH). There are other factors that may interfere with the normal release of GH from the pituitary, such as nutrient content, other hormones, stress and inflammatory factors [551].

Inflammation may dysregulate normal bone growth by altering secretions of some hormones and mineral content necessary for bone development. Through cytokine action, inflammation may exert a negative impact on bone remodeling. IL-1 was shown to contribute to bone resorption [552] and decreased secretion of GH [553].

The consumption of a high-fat diet may also interfere with bone growth by affecting the secretion of GH [554]. It has been shown that children fed a high-fat diet before exercise had reduced secretion of GH, suggesting that food high in calories may negatively interfere with normal growth and development [554].

The possible effect of single and repeated LPS inflammatory challenge and high-fat diet on longitudinal growth will be discussed in chapter 4.4.3.

4.1.4 Effect of biochemical parameters on dopamine circuit

As it was previously described in the general introduction, alteration in insulin and leptin levels may affect proper function of the dopamine circuitry. Under normal conditions, leptin and insulin are positive regulators of dopamine release from the TH-neurons. The release of dopamine from substantia nigra to the striatum is dependent on ATP-sensitive K^+ channels activity and its function is directly related to the level of glucose [361]. Since it has been shown that both hormones are involved in glucoregulation [360], it could be hypothesized that this process might be a connection of insulin and leptin-mediated dopamine secretion.

On the other hand, high levels of leptin and insulin may negatively influence the function of dopamine neurons. For example, leptin resistance in mice fed a high-fat diet has been related with decreased expression of TH enzyme [366]. Leptin and insulin insensitivity are suggested to influence down-regulation of D2 receptors in leptin-deficient obese rodents [368]. In particular, insulin resistance has been negatively correlated with dopamine release and its clearance from synaptic cleft in high-fat diet-fed mice [12].

The possible effect of single and repeated LPS inflammatory challenge and high-fat diet consumption on glucose tolerance, blood levels of insulin and leptin will be discussed in chapter 4.4.4.

4.2 Objectives

To determine the effect of single or repeated LPS inflammatory challenges and high-fat diet consumption on:

1) Food intake;
2) Body weight gain;
3) Longitudinal body growth, bone remodeling; and
4) Glucose tolerance and level of hormones such as leptin and insulin.

4.3 Brief methodology

1) Food intake was measured every week for seven months. At the end of every seven days, the leftover food from the cage was weighted and recorded. Food intake was expressed as an average of food weight (in kcal) per animal and normalized by animal body weight (BW).
2) The mouse`s body weight was measured weekly over a period of twelve months. Mice were weighted and their body weight was recorded. Final results were expressed as a mean value of total number of animals/per group. Towards the end of *in vivo* experiments, body composition (percentage of fat mass and weight of lean mass) was determined by DEXA scan.
3) The mouse`s body length (from anus to nose) was measured at the baseline and after 13 months. Towards the end of *in vivo* experiments, animal BMC and BMD were determined by DEXA scan.
4) At the end of 13 months starting from the first saline/LPS injection, mice were subjected to intraperitoneal glucose tolerance test in order to assess their

glucose tolerance according to their treatment. Two months later, mice were euthanized and their blood collected and plasma levels of leptin and insulin were assessed by ELISA method.

4.4 Results

4.4.1 Effect of LPS inflammatory challenge and high-fat diet on food intake

As it was previously described (chapter 4.1.1), food intake may be influenced by inflammation.

Figure 4.1 (A, C and E) shows averaged food intake (kcal/g) of WT, Casp-1-/- and IL-1ra-/- mice that was measured weekly over a period of seven months. The food intake of IL-1ra-/- mice was reported for saline and 5-times LPS-injected mice since the one time LPS-injected group was mixed with their heterozygote littermates in their home cage. As it may be observed in figure 4.1 (A, C and E) food intake was not affected by single or repeated LPS inflammatory challenges in any of the three genotypes.

Figures 4.1 (B, D and F) displays food intakes adjusted by body weight of each genotype and for each treatment (Sal, LPS-1T and LPS-5T). The comparison within each genotype revealed that repeated LPS inflammatory challenges significantly increased food intake in WT mice, whereas it was decreased in IL-1ra-/- mice (figure 4.1 B and F). On the other hand, neither single nor repeated LPS inflammatory challenges altered food intake in Casp-1-/- mice (figure 4.1 D).

Figure 4.1 G was constructed by collapsing all the results on food intake from figure 4.1 (A, C and E) in order to compare food intake among the three different genotypes within each treatment (Sal, LPS-1T and LPS-5T). Food intake of Casp-1-/- mice in the saline-treated group was significantly higher when compared to WT and IL-1ra-/- mice. Additionally, food intake in IL-1ra-/- mice was reduced with repeated LPS injections.

Food intake was normalized by body weight for each genotype and summarized in figure 4.1 H. IL-1ra-/- mice were shown to have increased food consumption per gram/BW versus both Casp-1-/- and WT mice in the saline-treated group. This trend was not observed in the five LPS-treated group, as food intake in WT mice tended to

increase, whereas repeated inflammatory challenge decreased food consumption in IL-1ra-/- mice.

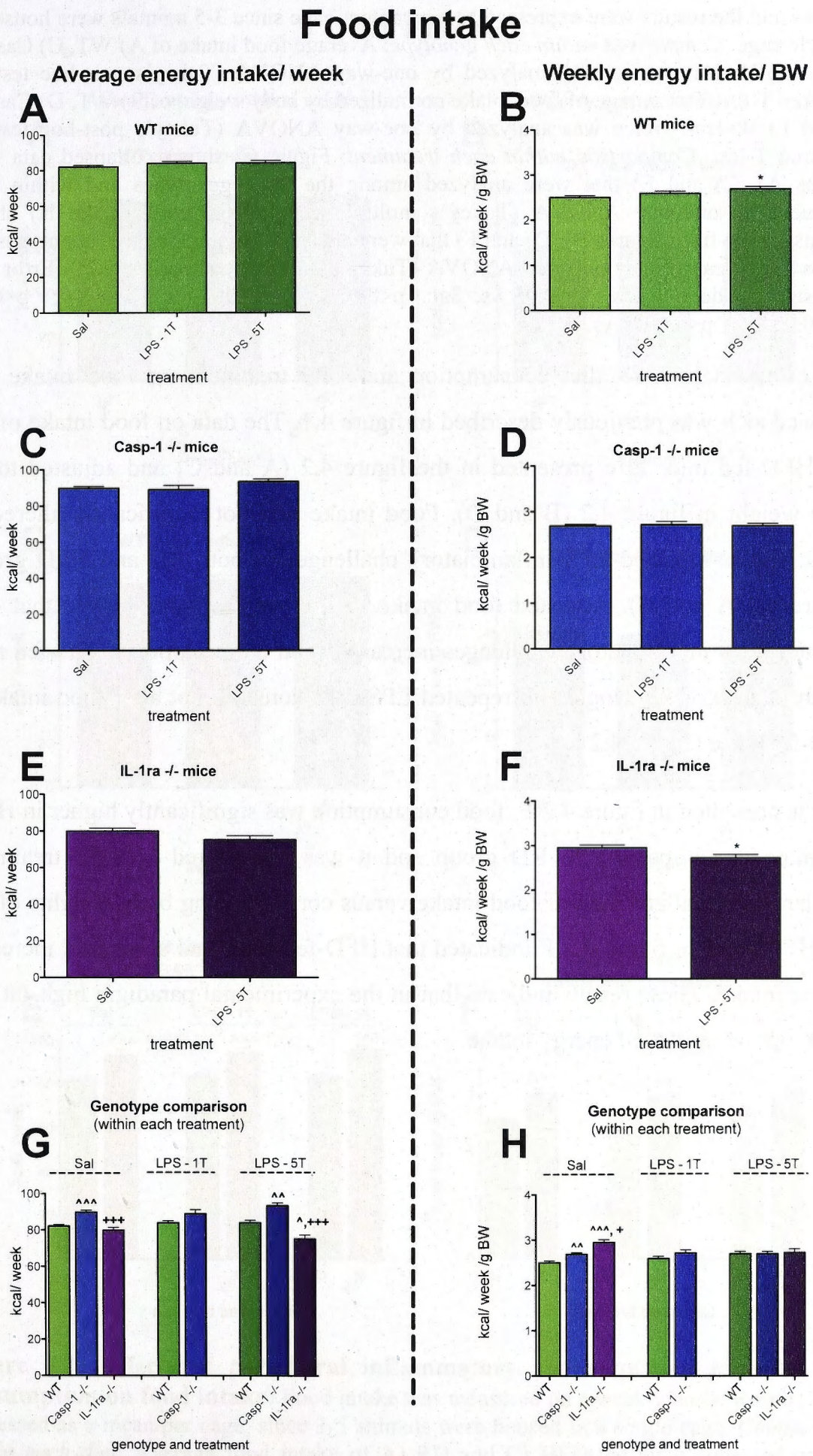


Figure 4.1: Effect of LPS inflammatory challenge on food intake: Food intake

(kcal) of WT, Casp-1-/- and IL-1ra-/- mice was measured on a weekly basis for seven months and the results were expressed as a mean/per cage since 3-5 animals were housed in a single cage. *Comparison within each genotype*: Average food intake of A) WT, C) Casp-1-/- and E) IL-1ra-/- mice was analyzed by one-way ANOVA (Tukey`s post-hoc test) or unpaired T-test. Percentage of food intake normalized by body weight of B) WT, D) Casp-1-/- and F) IL-1ra-/- mice was analyzed by one-way ANOVA (Tukey`s post-hoc test) or unpaired T-test. *Comparison within each treatment*: Figure G) shows collapsed data from figures A), C) and E) that were analyzed among the three genotypes and within each treatment by one-way ANOVA (Tukey`s multiple comparison test). Figure H) shows collapsed data from figures B), D) and F) that were analyzed among the three genotypes and within each treatment by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors. *$p<0.05$ vs. Sal; $^{+}p<0.05$, $^{+++}p<0.0001$ vs. Casp-1-/-; ^$p<0.05$, ^^$p<0.001$, ^^^$p<0.0001$ vs. WT.

The effect of high-fat diet consumption and LPS treatment on food intake was assessed as it was previously described in figure 4.1. The data on food intake of RD and HFD-fed mice arre presented in the figure 4.2 (A and C) and adjusted to the body weight in figure 4.2 (B and D). Food intake was not significantly altered by single and/or repeated LPS inflammatory challenges in both RD and HFD groups (figure 4.2 A and C). Adjusted food intake to the body weight showed that only repeated LPS inflammatory challenges increased food consumption in RD-fed mice (figure 4.2 B), while single and repeated LPS injections did not alter food intake in HFD-fed mice (figure 4.2 D).

As it is presented in figure 4.2 E, food consumption was significantly higher in HFD-fed mice in comparison to RD group and it was not altered by LPS treatment. Similarly, normalized data of food intake versus corresponding body weights of RD and HFD mice in figure 4.2 F indicated that HFD-fed mice had an overall increased calorie intake. These results indicate that in the experimental paradigm high-fat diet consumption increased energy intake.

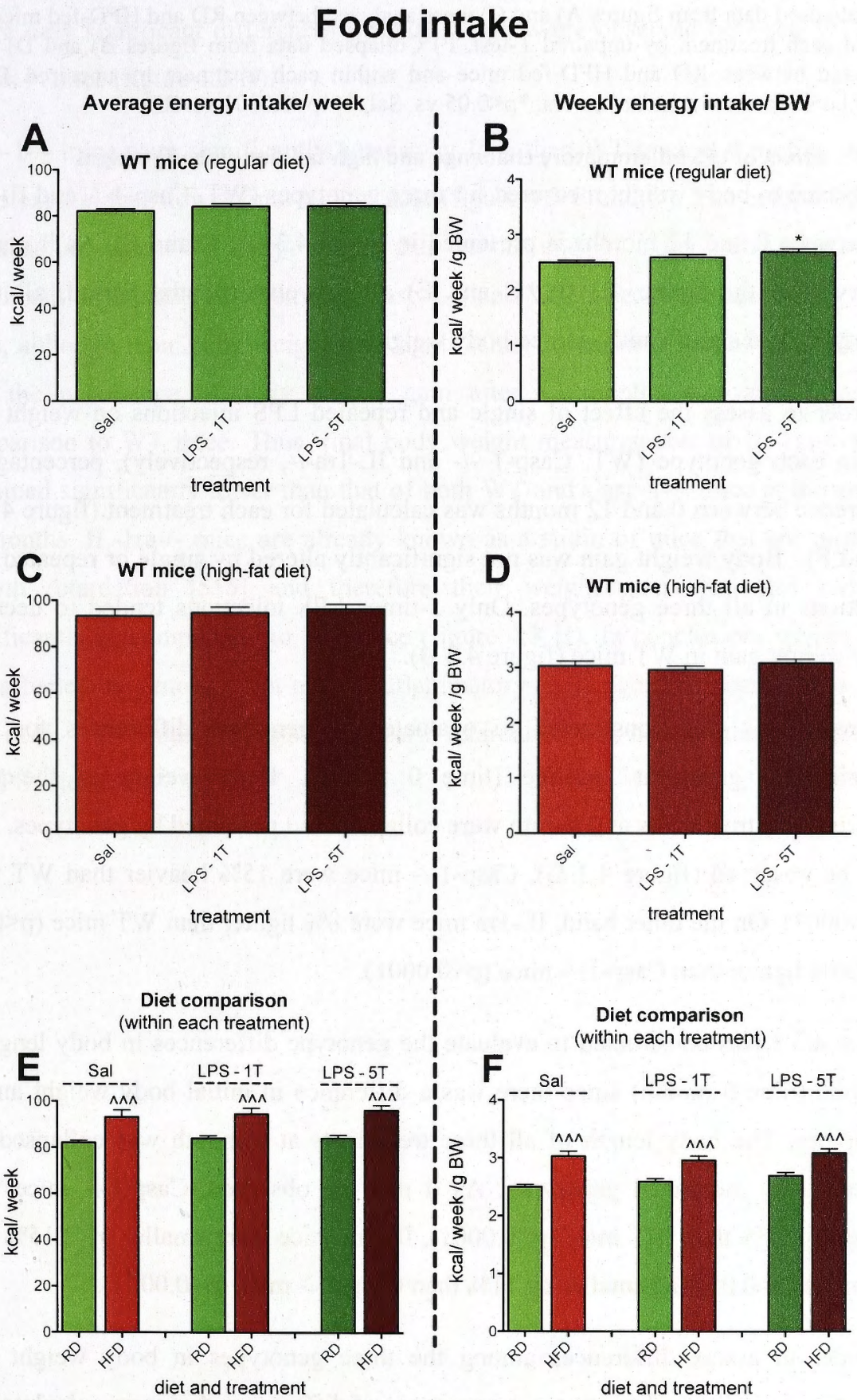


Figure 4.2: Effects of peripheral inflammatory challenge and high-fat diet consumption on food intake: Food intake was measured on a weekly basis. Results were expressed as a mean/per cage, since 3-5 animals were housed in a single cage. *Comparison within each diet*: Average food intake of A) RD and C) HFD-fed mice collected over a period of seven months was analyzed by one-way ANOVA (Tukey`s post-hoc test). Percentage of food intake normalized by body weight of B) RD and D) HFD-fed mice was

analyzed by one-way ANOVA (Tukey`s post-hoc test). *Comparison within each treatment*: E) Collapsed data from figures A) and C) were analyzed between RD and HFD-fed mice and within each treatment by unpaired T-test. F) Collapsed data from figures B) and D) were analyzed between RD and HFD-fed mice and within each treatment by unpaired T-test. Error bars represent standard errors. *p<0.05 vs. Sal, ^^^p<0.0001 vs. RD.

4.4.2 Effect of LPS inflammatory challenge and high-fat diet on body weight

Difference in body weight measured for three genotypes (WT, Casp-1-/- and IL-1ra-/-) between 0 and 12 months is presented in figure 4.3 (A, C and E). As it may be observed in the figure 4.3 (A, C and E) after twelve months period, all three genotypes had significantly gained body weight.

In order to assess the effect of single and repeated LPS injections on weight gain within each genotype (WT, Casp-1 -/- and IL-1ra-/-, respectively), percentage of difference between 0 and 12 months was calculated for each treatment (figure 4.3 B, D and F). Body weight gain was not significantly altered by single or repeated LPS injections in all three genotypes. Only 5-times LPS injections tended to decrease body weight gain in WT mice (figure 4.3 B).

Figure 4.3 G was constructed to evaluate the genotype differences for each experimental group at baseline (time 0 month). Body weight of the three experimental treatments at 0 month were collapsed and presented by genotypes. As it may be observed (figure 4.3 G), Casp-1-/- mice were 15% heavier than WT mice ($p<0.0001$). On the other hand, IL-1ra mice were 8% lighter than WT mice ($p<0.05$) and 20% lighter than Casp-1-/- mice ($p<0.0001$).

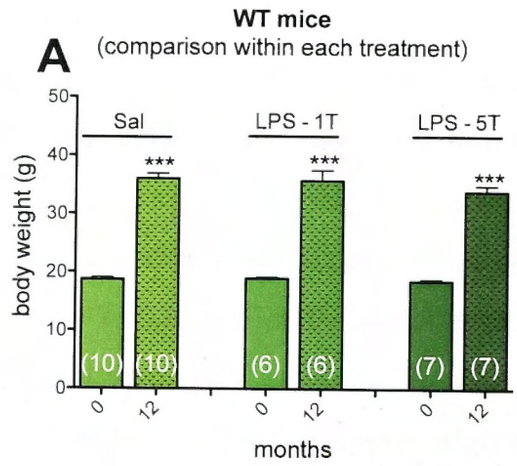
Figure 4.3 I was constructed to evaluate the genotype differences in body length at baseline (time 0 month) since there was a difference in initial body weight among genotypes. The body length of all three treatments at 0 month was collapsed and presented by individual genotypes. As it may be observed, Casp-1-/- mice were longer by 15% than WT mice ($p<0.0001$). IL-1ra mice were smaller by 2.15% than WT mice ($p<0.05$) and smaller by 11% than Casp-1-/- mice ($p<0.0001$).

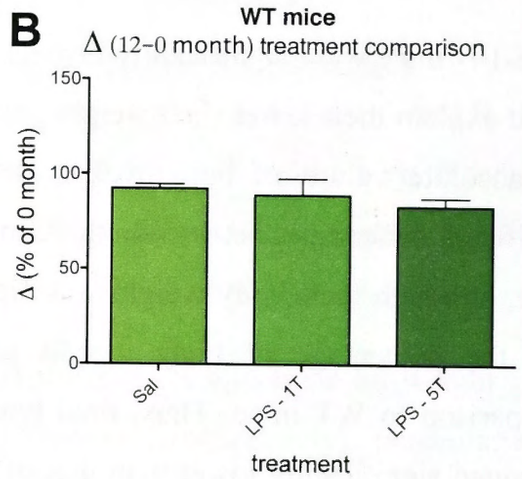
In order to assess differences among the three genotypes in body weight gain according to individual treatment, percentages of differences that were calculated for each genotype in figure 4.3 (B, D and F) were collapsed in the figure 4.3 H. The comparison among saline-treated group revealed that weight gain of Casp-1-/- and IL-1ra-/- mice were significantly lower than that of WT mice (figure 4.3 H). A

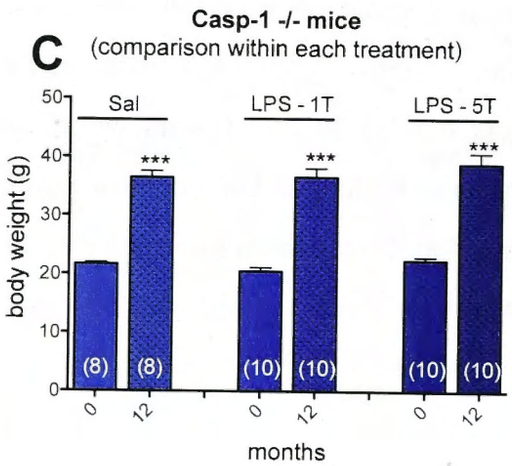
similar trend was observed in groups injected with single or repeated LPS injections, where only significant differences in body weight were observed between WT and IL-1ra-/- mice (figure 4.3 H).

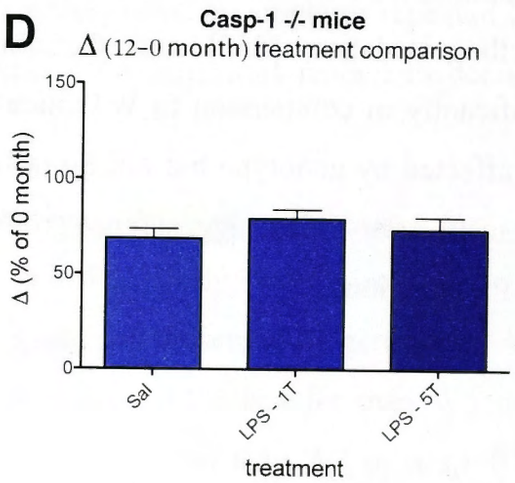
Casp-1-/- mice were significantly heavier by 15% than WT mice at 0 month, which could explain their lower final weight gain (figure 4.3 C and D). The comparison of the absolute values of body weight data at the end of experiment showed no significant differences between both Casp-1-/- and WT mice. In the case of IL-1ra-/- mice, although their body weight was significantly lower than that of WT mice by 8%, the percentage of body weight gain after 12 months was also lower in comparison to WT mice. Thus, final body weight measurements of IL-1ra-/- mice remained significantly lower than that of both WT and Casp-1-/- mice at the end of 12 months. IL-1ra-/- mice are already known as a strain of mice that are prone to growth retardation [555] and therefore their weight gain was also reduced significantly in comparison to WT mice (figure 4.3 H). In conclusion, weight gain was affected by genotype but not by inflammatory challenge. The decreased weight gain in IL-1ra-/- mice suggests that growth retardation was not additionally affected by LPS injections.

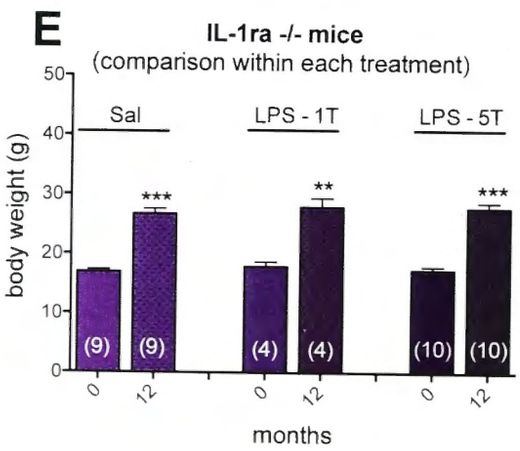
Body weight

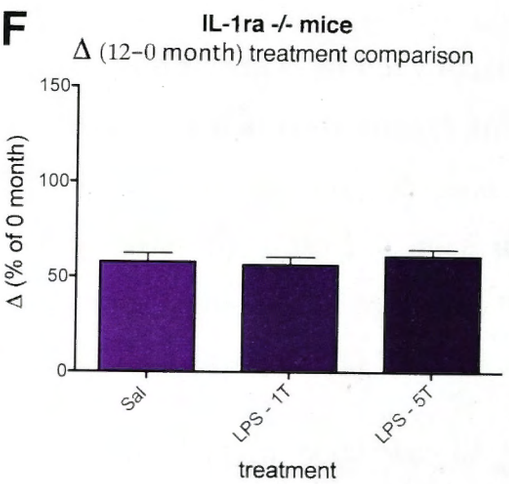

A
WT mice
(comparison within each treatment)
Sal
LPS - 1T
LPS - 5T
body weight (g)
***
(10) (10) (6) (6) (7) (7)
0 12 0 12 0 12
months


B
WT mice
Δ (12–0 month) treatment comparison
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
treatment


C
Casp-1 -/- mice
(comparison within each treatment)
Sal
LPS - 1T
LPS - 5T
body weight (g)
***
(8) (8) (10) (10) (10) (10)
0 12 0 12 0 12
months


D
Casp-1 -/- mice
Δ (12–0 month) treatment comparison
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
treatment


E
IL-1ra -/- mice
(comparison within each treatment)
Sal
LPS - 1T
LPS - 5T
body weight (g)
***
**
***
(9) (9) (4) (4) (10) (10)
0 12 0 12 0 12
months


F
IL-1ra -/- mice
Δ (12–0 month) treatment comparison
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
treatment

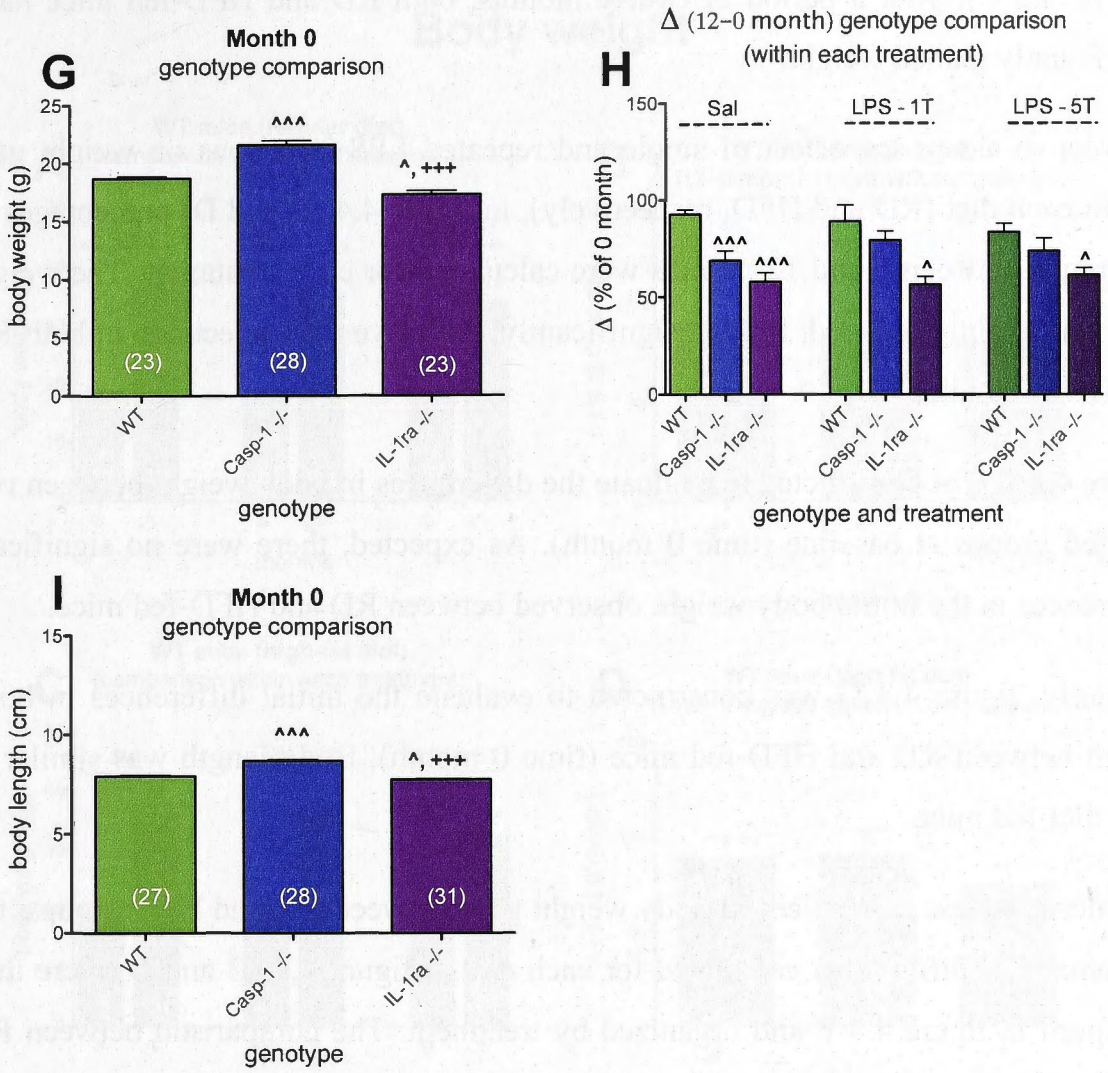


Figure 4.3: Effect of LPS inflammatory challenge on body weight gain: *Comparison within each genotype*: Absolute values of body weight data of A) WT, C) Casp-1-/- and E) IL-1ra-/- mice were measured at 0 and 12 months and compared within each treatment by paired T-test. Final weight gain of B) WT, D) Casp-1-/- and F) IL-1ra-/- mice was normalized versus initial baseline weight and expressed as percentage of difference, calculated by the formula previously described in the legend to figure 3.1. Data were analyzed by one-way ANOVA (Tukey`s multiple comparison test). G) Initial body weight data measured at 0 month were collapsed from figures A), C) and E) and analyzed by one-way ANOVA followed by Tukey`s post-hoc test. I) Body lengths of three genotypes measured at 0 month were analyzed by one-way ANOVA followed by Tukey`s post-hoc test. *Comparison within each treatment:* H) Percentages of differences in body weight gain were compared among genotypes within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. **p<0.001, ***p<0.0001 vs. 0 month; ^p< 0.05, ^^^p< 0.0001 vs. WT; $^{+++}$p<0.0001 vs. Casp-1-/-.

In order to ascertain the effect of high-fat diet consumption and LPS treatment on metabolic outcomes, body weight gain was determined in mice fed a RD and HFD.

The body weights of RD and HFD-fed mice are presented in the figure 4.4 (A and C) showing body weight gain between 0 and 12 months. As it may be observed in figure

4.4 (A and C), after a period of twelve months, both RD and HFD-fed mice have significantly gained weight.

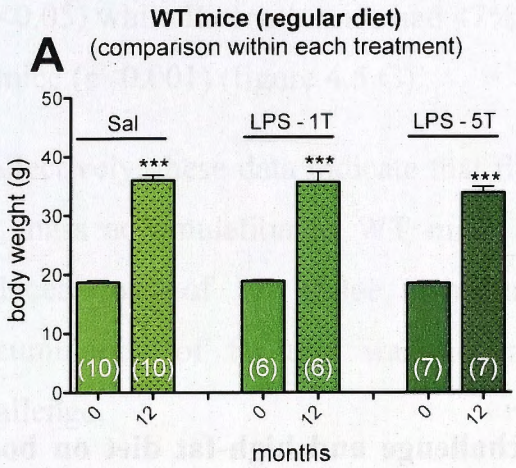
In order to assess the effect of single and repeated LPS injections on weight gain within each diet (RD and HFD, respectively), in figure 4.4 (B and D) percentages of difference between 0 and 12 months were calculated for each treatment. The weight gain was slightly reduced, but not significantly, with five LPS injections in both RD and HFD-fed mice.

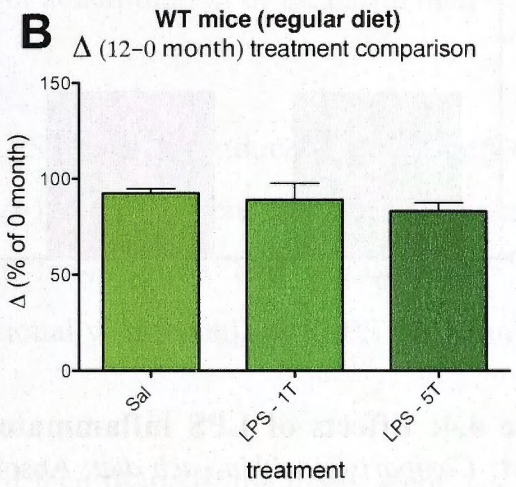
Figure 4.4 E was constructed to evaluate the differences in body weight between two diet-fed groups at baseline (time 0 month). As expected, there were no significant differences in the initial body weight observed between RD and HFD-fed mice.

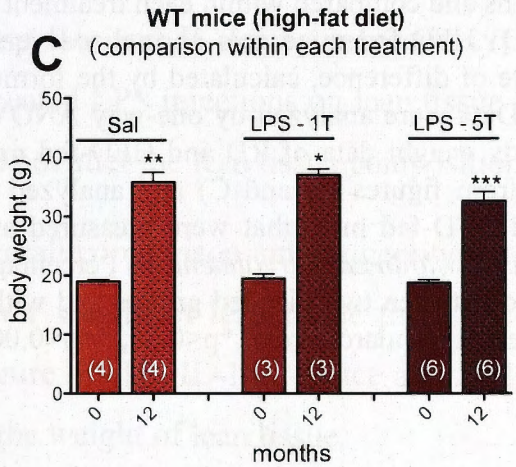
Similarly, figure 4.4 G was constructed to evaluate the initial differences in body length between RD and HFD-fed mice (time 0 month). Body length was similar in both diet-fed mice.

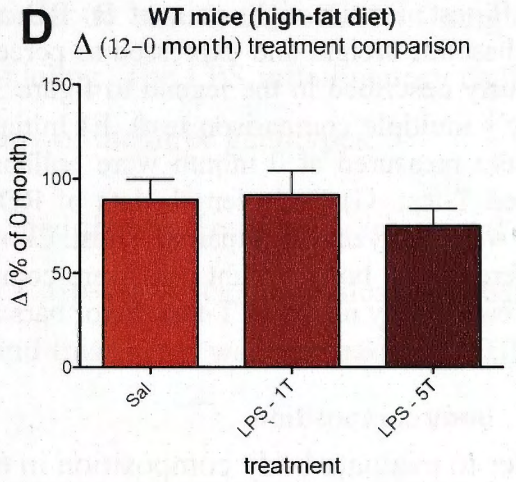
In order to assess differences in body weight gain between RD and HFD groups, the percentage of differences calculated for each diet in figure 4.4 (B and D) were then collapsed in figure 4.4 F and organized by treatment. The comparison between RD and HFD-fed mice indicated that body weight gain was similar in both groups.

Body weight

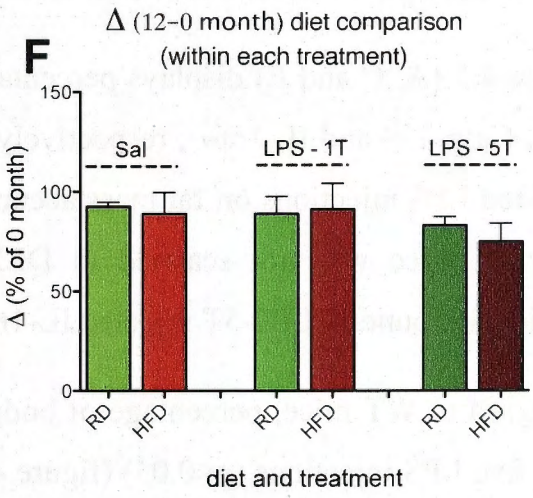

A
WT mice (regular diet)
(comparison within each treatment)
Sal
LPS - 1T
LPS - 5T
body weight (g)
***
(10) (10) (6) (6) (7) (7)
0 12 0 12 0 12
months


B
WT mice (regular diet)
Δ (12–0 month) treatment comparison
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
treatment


C
WT mice (high-fat diet)
(comparison within each treatment)
Sal
LPS - 1T
LPS - 5T
body weight (g)
**
*
***
(4) (4) (3) (3) (6) (6)
0 12 0 12 0 12
months


D
WT mice (high-fat diet)
Δ (12–0 month) treatment comparison
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
treatment


E
Month 0
diet comparison
body weight (g)
(23)
(13)
RD
HFD
diet


F
Δ (12–0 month) diet comparison
(within each treatment)
Δ (% of 0 month)
Sal
LPS - 1T
LPS - 5T
RD
HFD
diet and treatment

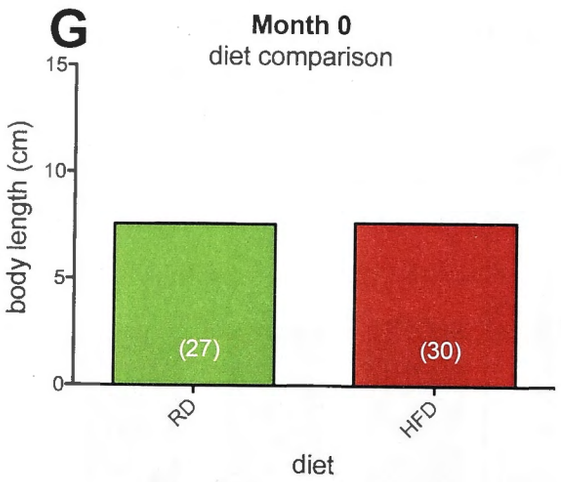


Figure 4.4: Effects of LPS inflammatory challenge and high-fat diet on body weight: *Comparison within each diet*: Absolute values of body weight data of A) RD and C) HFD-fed mice were measured at 0 and 12 months and compared within each treatment by paired T-test. Final weight gain of B) RD and D) HFD-fed mice was normalized versus initial baseline weight and expressed as percentage of difference, calculated by the formula previously described in the legend to figure 3.1. Data were analyzed by one-way ANOVA (Tukey`s multiple comparison test). E) Initial body weight data of RD and HFD-fed mice that were measured at 0 month were collapsed from figures A) and C) and analyzed by unpaired T-test. G) Body length data of RD and HFD-fed mice that were measured at 0 month were analyzed by unpaired T-test. *Comparison within each treatment*: F) Percentages of differences in body weight gain were compared between two diet-fed groups and within each treatment by unpaired T-test. Error bars represent standard errors. *p<0.05, **p<0.001, ***p<0.0001 vs. 0 month.

4.4.3 Body composition

In order to evaluate body composition in the three genotypes, the percentage of body fat mass, weight of lean mass, BMD and BMC were assessed with DEXA scan.

Figure 4.5 (A, C and E) displays percentages of body weight of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) in order to assess the effect of single and repeated LPS injections on fat mass accumulation. The group of single LPS injected IL-1ra-/- mice was not scanned in DEXA apparatus since no differences were previously found in LPS-5T injected IL-1ra-/- group.

In regard to WT mice, percentage of body fat mass significantly decreased by 28% with five LPS injections ($p<0.05$) (figure 4.5 A). However, accumulation of fat mass was not affected with single and repeated LPS injections in Casp-1-/- and IL-1-ra -/- mice (figure 4.5 C and E).

Comparison among genotypes and within saline-treated groups indicated that accumulation of fat mass in IL-1ra-/- mice was significantly lower by 43% in comparison to WT mice ($p<0.01$) and by 45% in comparison to Casp-1-/- mice

($p<0.01$) (figure 4.5 G). In 5-times LPS-injected groups, the percentage of fat tissue in WT mice was significantly lower by 37% in comparison to Casp-1-/- mice ($p<0.05$) while IL-1ra-/- mice had 47% lower accumulation of fat tissue than Casp-1-/- mice ($p<0.001$) (figure 4.5 G).

Collectively, these data indicate that five LPS injections induced significantly lower fat mass accumulation in WT mice. Casp-1-/- mice were protected against LPS induced loss of fat tissue, whereas IL-1ra-/- mice had significantly lower accumulation of fat that was not additionally affected by LPS inflammatory challenge.

Figure 4.5 (B, D and F) shows the weight of lean tissue of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) in order to assess the effect of single and repeated LPS injections on lean tissue remodeling. The LPS inflammatory challenge did not alter the lean tissue composition in any of the three genotypes.

Overall comparison among genotypes indicated that IL-1ra-/- mice in general had a lower weight of lean tissue than WT and Casp-1-/- mice, regardless of treatment (figure 4.5 H). IL-1ra-/- mice are smaller and this factor was subsequently reflected in the weight of lean tissue.

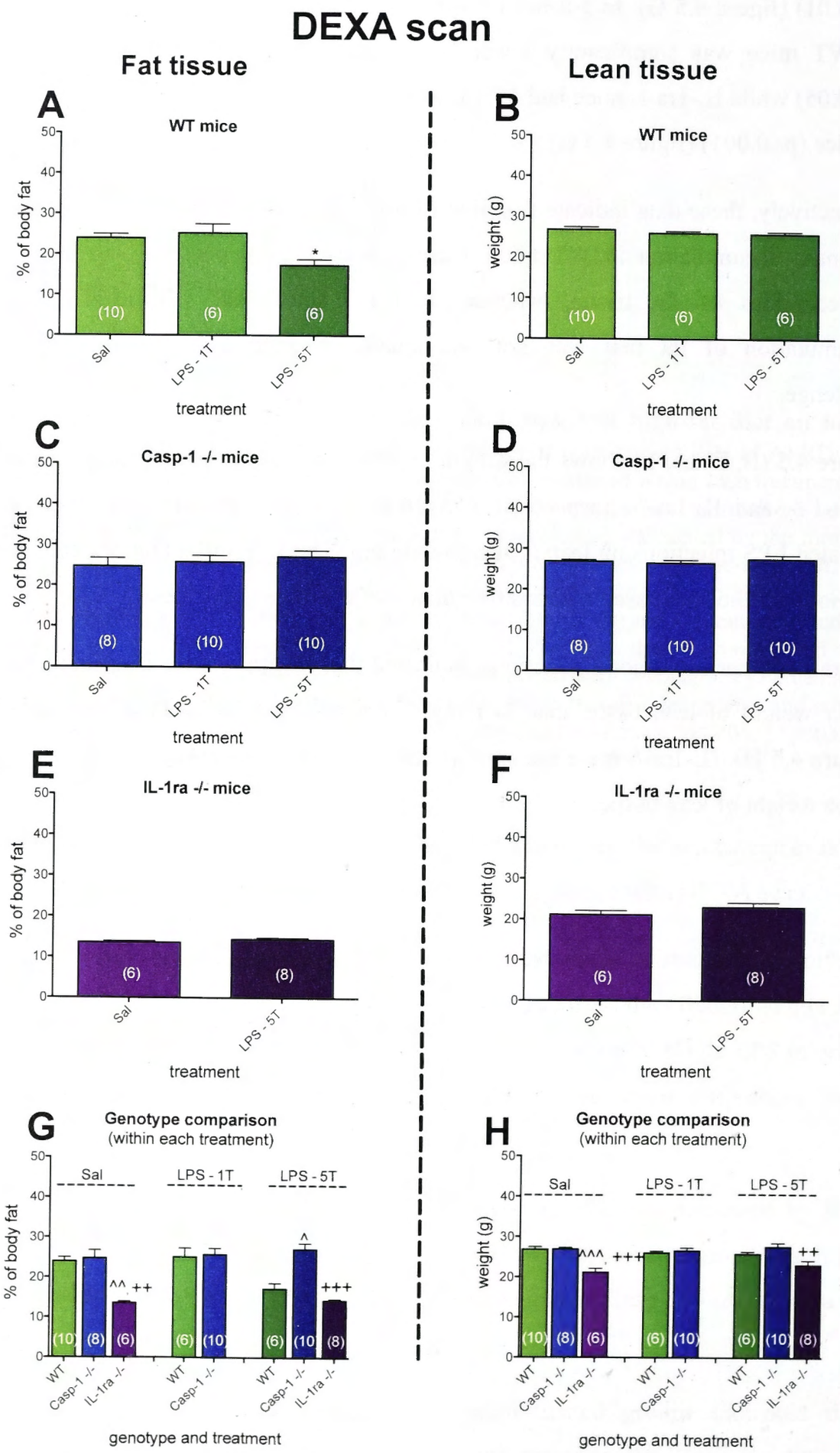


Figure 4.5: Effect of LPS inflammatory challenge on body fat mass and lean

tissue remodeling: *Comparison within each genotype*: Percentage of body fat mass of A) WT C) Casp-1-/- and E) IL-1ra-/- mice was analyzed by one-way ANOVA (Tukey`s multiple comparison test) or unpaired T-test. Weight of lean mass of B) WT, D) Casp-1-/- and F) IL-1ra-/- mice was analyzed by one-way ANOVA (Tukey`s multiple comparison test) or unpaired T-test. *Comparison within each treatment*: G) Percentages of body fat mass were analyzed among genotypes and within each treatment by one-way ANOVA (Tukey`s multiple comparison test). H) Weight of lean mass was analyzed among genotypes and within each treatment by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors. *$p<0.05$ vs. Sal; ^$p<0.05$, ^^$p<0.01$, and ^^^$p<0.001$ vs. WT; $^{++}p<0.01$ and $^{+++}p<0.001$ vs. Casp-1-/-.

The bone analysis was further performed by DEXA scan, using parameters such as BMD and BMC.

Figure 4.6 (A, D and G) shows the final body length of three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) measured 13 months after the first saline/LPS injection. The body length was not affected by single and/or repeated LPS injections in any of the three genotypes.

Figure 4.6 J displays the body length of all three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively), in order to evaluate the differences among the genotypes within each treatment. Genotype comparison revealed that IL-1ra-/- mice had shown retardation in longitudinal growth, regardless of treatment and this was significantly different by 13% versus WT ($p<0.0001$) and by 15% versus Casp-1-/- mice ($p<0.0001$).

Figure 4.6 (B, E and H) displays the data on BMC of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) in order to assess in more detail the effect of LPS treatment on bone composition. As it may be observed in the figure 4.6 B, LPS inflammatory challenge decreased BMC in WT mice and this loss was significant by 18% with five LPS injections ($p<0.05$). Similarly, single LPS injection reduced BMC by 26% ($p<0.0001$) and by 16% with repeated LPS injections ($p<0.001$) in Casp-1-/- mice (figure 4.6 E). Repeated LPS injections did not reduce BMC in IL-1ra-/- mice (figure 4.6 H).

Evaluation of BMC among the three genotypes in saline-treated groups (figure 4.6 K) showed that Casp-1-/- mice had significantly higher BMC by 23% in comparison to WT ($p<0.0001$) and by 33% versus IL-1ra-/- mice ($p<0.0001$). The IL-1ra-/- mice had the least BMC in comparison to other two genotypes, by 15% lower versus WT mice ($p<0.001$). Single LPS injection reduced BMC in WT and Casp-1-/- mice.

Repeated LPS injections reduced the BMC of WT and Casp-1-/- mice to the similar level as it observed in IL-1ra-/- mice, but the differences between WT and Casp-1-/- mice still remained. In summary, longitudinal growth of IL-1ra-/- mice was related with BMC. Casp-1-/- mice had increased BMC, which was not reflected in body length.

Figure 4.6 (C, F and I) displays the data of BMD of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively), in order to assess the effect of LPS inflammatory challenge on bone resorption. As it may be observed in figure 4.6 C, LPS treatment decreased the BMD in WT mice and it reached significance in five LPS-injected group. Repeated LPS injections reduced BMD by 18% versus WT-Sal ($p<0.0001$) and by 16% versus WT-LPS-1T ($p<0.001$). Similarly, single LPS injection reduced BMD by 25% ($p<0.0001$) and by 15% with repeated LPS injections in Casp-1-/- mice (figure 4.6 F). Repeated LPS injections did not reduce BMD in IL-1ra-/- mice (figure 4.6 I).

Evaluation of BMD among the three genotypes in saline-treated groups (figure 4.6 L) showed that Casp-1-/- mice had significantly higher BMD by 12.5% in comparison to WT ($p<0.001$) and by 25% versus IL-1ra-/- mice ($p<0.0001$). Single LPS injection reduced BMD in WT and Casp-1-/- mice. Repeated LPS injections reduced the BMD of WT and Casp-1-/- mice to the similar level as it observed in IL-1ra-/- mice, but the differences between WT and Casp-1-/- mice still remained. Overall, repeated systemic LPS inflammatory challenges reduced BMD in WT and Casp-1-/- mice and, in particular, reduction in BMD of Casp-1-/- mice was also observed with single LPS injection.

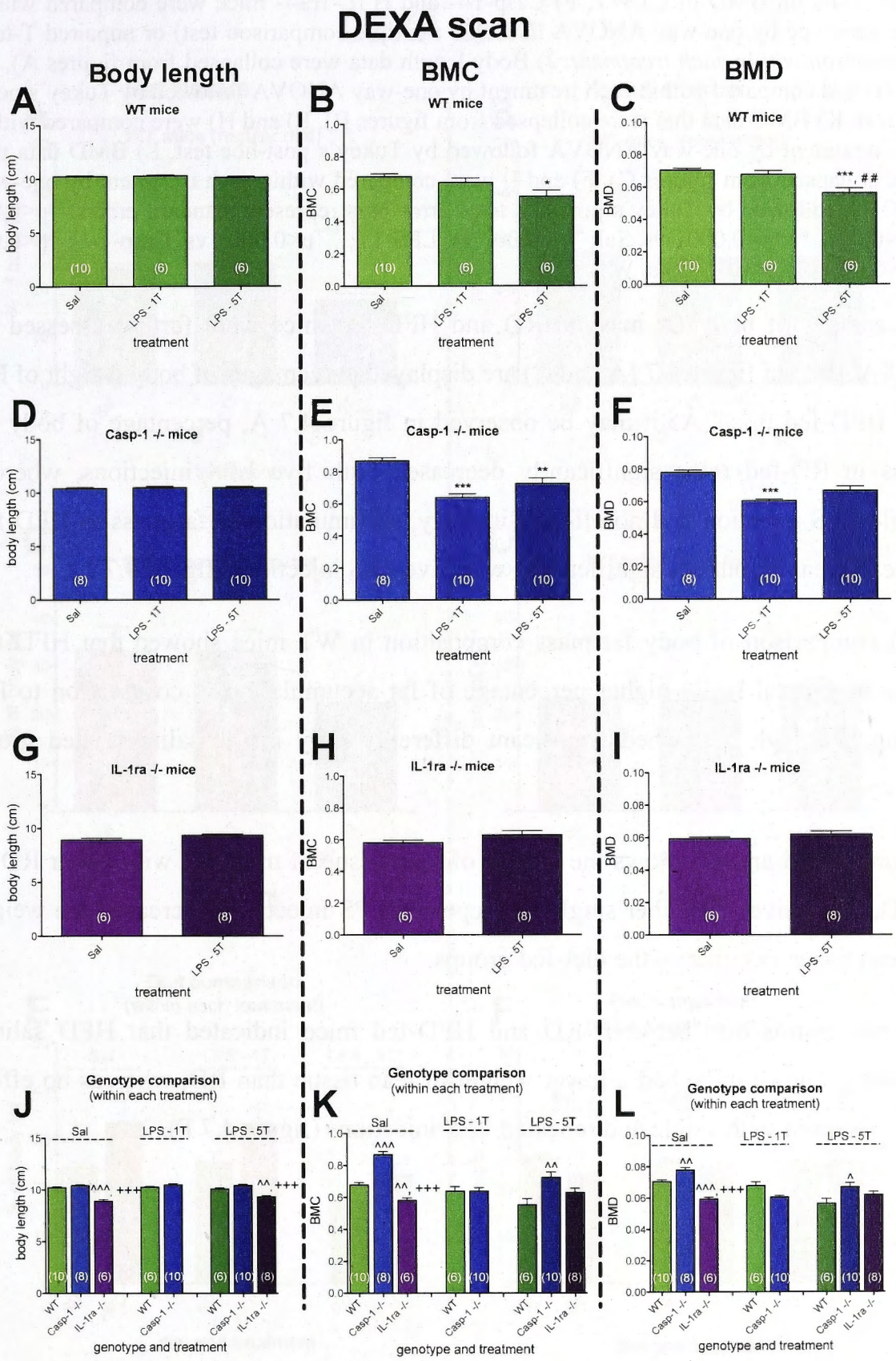


Figure 4.6: Effect of LPS inflammatory challenge on body length, BMC and BMD: Body length, BMC and BMD were measured thirteen months after the first saline/LPS injection by DEXA scan. *Comparison within each genotype*: Absolute body length values of A) WT D) Casp-1-/- and G) IL-1ra-/- mice were measured at 13 months and compared within each genotype by one-way ANOVA (Tukey`s multiple comparison test) or unpaired T-test. Data on BMC of B) WT, E) Casp-1-/- and H) IL-1ra-/- mice were compared within each genotype by one-way ANOVA (Tukey`s multiple comparison test) or unpaired

T-test. Data on BMD of C) WT, F) Casp-1-/- and I) IL-1ra-/- mice were compared within each genotype by one-way ANOVA (Tukey`s multiple comparison test) or unpaired T-test. *Comparison within each treatment*: J) Body length data were collapsed from figures A), D) and G) and compared within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. K) BMC data that were collapsed from figures B), E) and H) were compared within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. L) BMD data that were collapsed from figures C), F) and I) were compared within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. *$p<0.05$, **$p<0.001$, ***$p<0.0001$ vs. Sal; $^{\#\#}p<0.001$ vs. LPS-1T; $^{+++}p<0.0001$ vs. Casp-1-/-; ^$p<0.05$, ^^$p<0.001$, ^^^$p<0.0001$ vs. WT.

Percentages of body fat mass in RD and HFD-fed mice were further assessed by DEXA scan. In figure 4.7 (A and C) are displayed percentages of body weight of RD and HFD-fed mice. As it may be observed in figure 4.7 A, percentage of body fat mass in RD-fed mice significantly decreased with five LPS injections, whereas single LPS injection had no effect. Similarly, accumulation of fat mass in HFD-fed mice decreased, but not significantly, with five LPS injections (figure 4.7 C).

Diet comparison of body fat mass composition in WT mice showed that HFD-fed mice in general had a higher percentage of fat accumulation in comparison to RD group, although it reached significant difference only in the saline-treated group (figure 4.7 E).

Figure 4.7 (B and D) shows the weight of lean tissue of mice fed with either RD or HFD, respectively. Neither single nor repeated LPS injections decreased the weight of lean tissue in either of the diet-fed groups.

Overall comparison between RD and HFD-fed mice indicated that HFD saline-treated group of mice had a lower weight of lean tissue than RD, whereas no effect was observed with single and repeated LPS injections (figure 4.7 F).

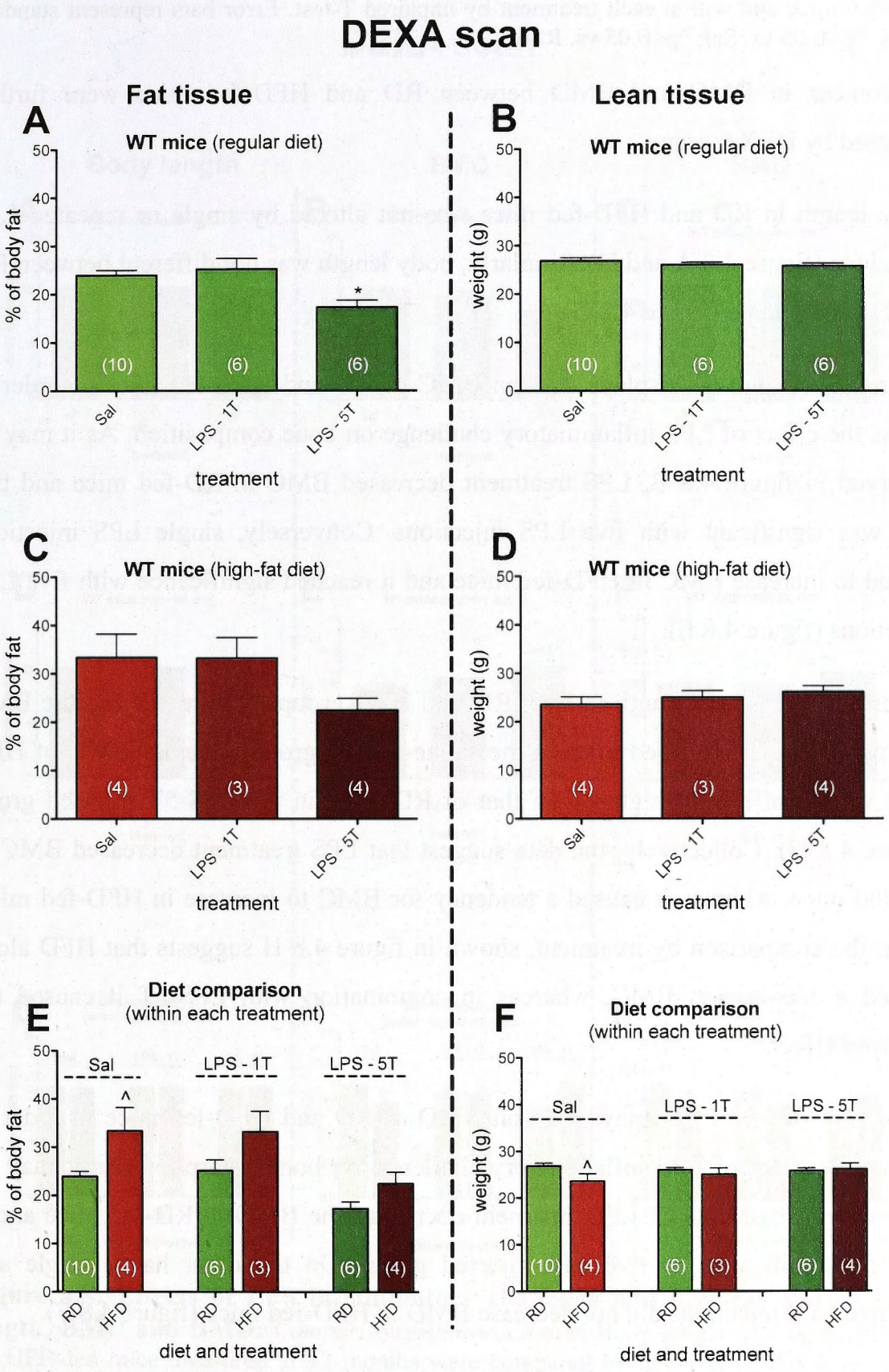


Figure 4.7: Effects of LPS inflammatory challenge and high-fat diet on fat mass and lean tissue remodeling: *Comparison within each diet*: Percentages of body fat mass of A) RD and C) HFD-fed mice were analyzed by one-way ANOVA (Tukey`s multiple comparison test). Weight of lean tissue of B) RD and D) HFD-fed mice was analyzed by one-way ANOVA (Tukey`s multiple comparison test). *Comparison within each treatment*: E) Percentages of body fat mass were analyzed between RD and HFD-fed mice and within each treatment by unpaired T-test. F) Weight of lean tissue was analyzed between RD and

HFD-fed mice and within each treatment by unpaired T-test. Error bars represent standard errors. *p<0.05 vs. Sal; ^p<0.05 vs. RD.

Differences in BMC and BMD between RD and HFD-fed mice were further analyzed by DEXA scan.

Body length in RD and HFD-fed mice was not altered by single or repeated LPS injections (figure 4.8 A and D). Similarly, body length was not different between RD and HFD-fed mice (figure 4.8 G).

Figure 4.8 (B and E) displays data on BMC of RD and HFD-fed mice in order to assess the effect of LPS inflammatory challenge on bone composition. As it may be observed in figure 4.8 B, LPS treatment decreased BMC in RD-fed mice and this loss was significant with five LPS injections. Conversely, single LPS injections tended to increase BMC in HFD-fed mice and it reached significance with five LPS injections (figure 4.8 E).

Despite similar body length in both RD and HFD groups (figure 4.8 G), the BMC was decreased in HFD-fed mice in the saline-treated group, whereas BMC of HFD mice was significantly higher than that of RD mice in the LPS-5T injected group (figure 4.8 H). Collectively, the data suggest that LPS treatment decreased BMC in RD-fed mice, whereas it caused a tendency for BMC to increase in HFD-fed mice. Thus, the comparison by treatment, shown in figure 4.8 H suggests that HFD alone caused a decrease in BMC, whereas in combination with LPS-5T it caused the opposite effect.

Figure 4.8 (C and F) displays data on BMD of RD and HFD-fed mice in order to assess the effect of LPS inflammatory challenge on bone resorption. As it may be observed in figure 4.8 C, LPS treatment decreased the BMD in RD-fed mice and it reached significance in five LPS-injected group. On the other hand, single and repeated LPS injections did not decrease BMD in HFD-fed mice (figure 4.8 F).

Similar to the results of BMC (figure 4.8 H), the BMD was decreased in HFD-fed mice in the saline-treated group, whereas BMD of HFD mice was significantly higher than that of RD mice in the LPS-5T injected group (figure 4.8 I).

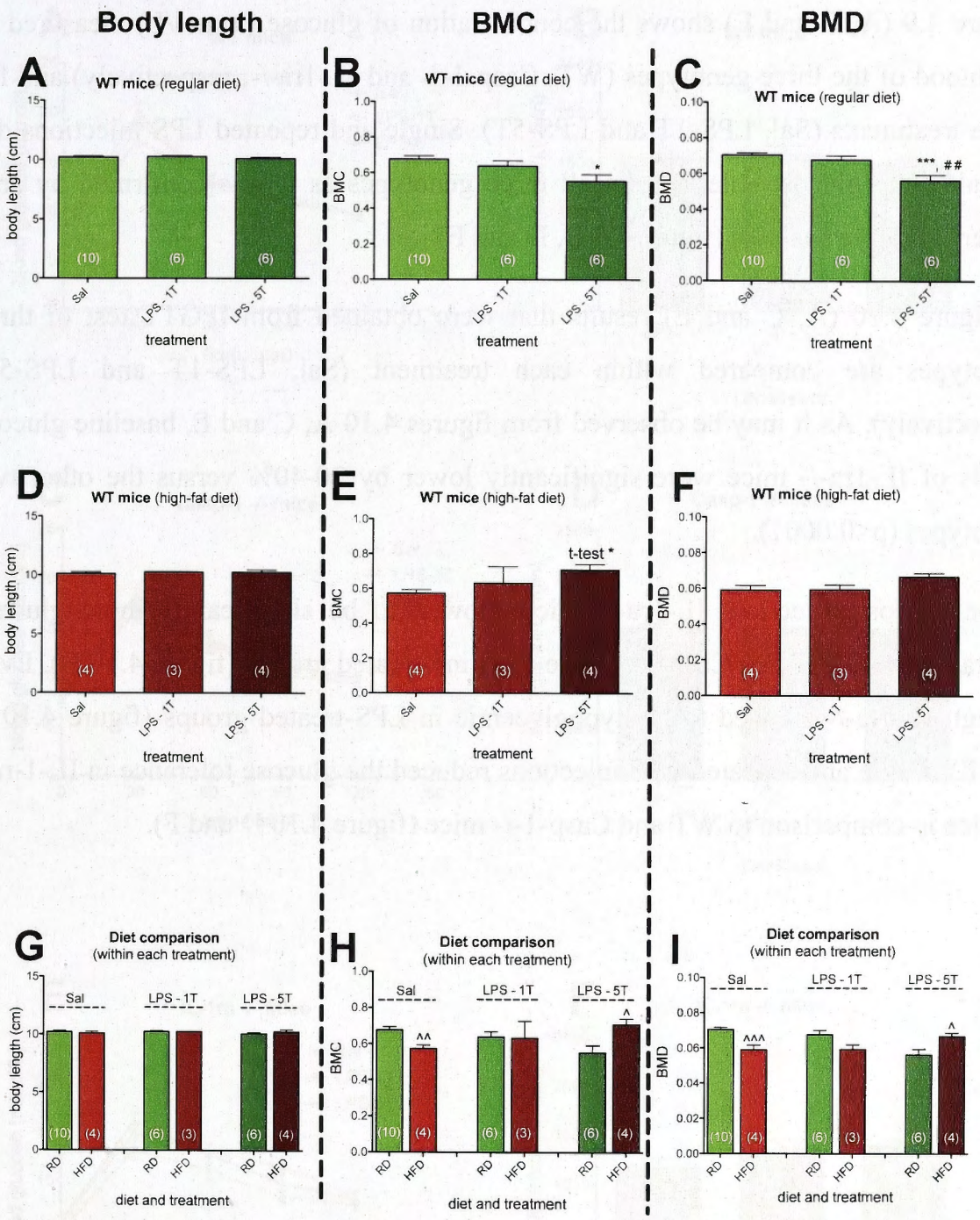


Figure 4.8: Effects of LPS inflammatory challenge and high-fat diet on body length, BMC and BMD: *Comparison within each diet*: Body length data of A) RD and D) HFD-fed mice measured at 13 months were compared by one-way ANOVA (Tukey`s multiple comparison test). Data on BMC of B) RD and E) HFD-fed mice were compared by one-way ANOVA (Tukey`s multiple comparison test) unless otherwise indicated. Data on BMD of C) RD and F) HFD-fed mice were compared by one-way ANOVA (Tukey`s multiple comparison test). *Comparison within each treatment:* G) Body length data were collapsed from figures A) and D) and compared within each treatment by unpaired T-test. H) Data on BMC were collapsed from figures B) and E) and compared within each treatment by unpaired T-test. I) Data on BMD were collapsed from figures C) and F) and compared within each treatment by unpaired T-test. Error bars represent standard errors. *$p<0.05$, ***$p<0.001$ vs. Sal; $^{\#\#}p<0.001$ vs. LPS-1T; ^$p<0.05$, ^^$p<0.01$, ^^^$p<0.001$ vs. RD.

4.4.4 Effect of LPS and high-fat diet on IPGTT, plasma insulin and leptin levels

Blood glucose level was determined in three genotypes (WT, Casp-1-/- and IL-1ra-/-) as they were subjected to IPGTT test at the end of 15 months of *in vivo* studies.

Figure 4.9 (A, C and E) shows the concentration of glucose (mmol/L) measured in the blood of the three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) and for three treatments (Sal, LPS-1T and LPS-5T). Single and repeated LPS injections did not alter the glucose tolerance in all three genotypes as it was confirmed by area under the curve analysis (figure 4.9 B, D and F).

In figure 4.10 (A, C and E) results that were obtained from IPGTT test of three genotypes are compared within each treatment (Sal, LPS-1T and LPS-5T, respectively). As it may be observed from figures 4.10 A, C and E, baseline glucose levels of IL-1ra-/- mice were significantly lower by 20-40% versus the other two genotypes ($p<0.0001$).

Upon glucose injection, IL-1ra-/- mice showed to be significantly more glucose tolerant versus WT and Casp-1-/- mice in saline-treated groups (figure 4.10 B). Even though IL-1ra-/- showed to be hypoglycemic in LPS-treated groups (figure 4.10 C and E), single and repeated LPS injections reduced the glucose tolerance in IL-1-ra -/- mice in comparison to WT and Casp-1-/- mice (figure 4.10 D and F).

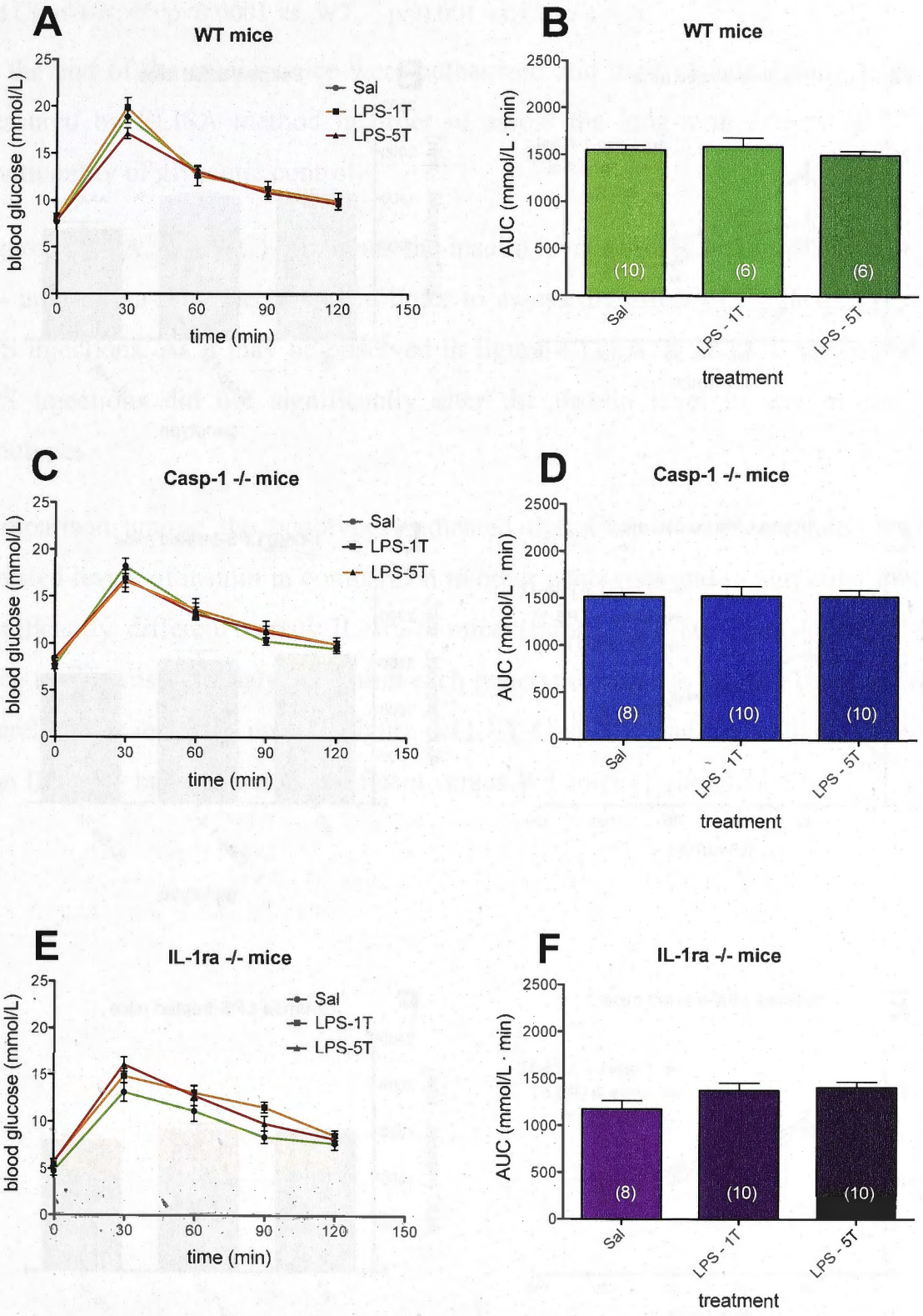


Figure 4.9: Effect of LPS inflammatory challenge on blood glucose level in IPGTT test: Mice were fasted for 6 hours and were injected (i.p.) with 2g/kg of glucose solution [393]. Blood glucose level was measured at 0 min before injection and 30, 60, 90 and 120 min after the injection. *Comparison within each genotype*: Blood glucose levels of A) WT, C) Casp-1-/- and E) IL-1ra-/- mice were analyzed by one-way ANOVA (Tukey`s multiple comparison test). Glucose tolerance in B) WT, D) Casp-1-/- and F) IL-1ra-/- mice

was analyzed by area under the curve (AUC) and compared within each genotype by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors.

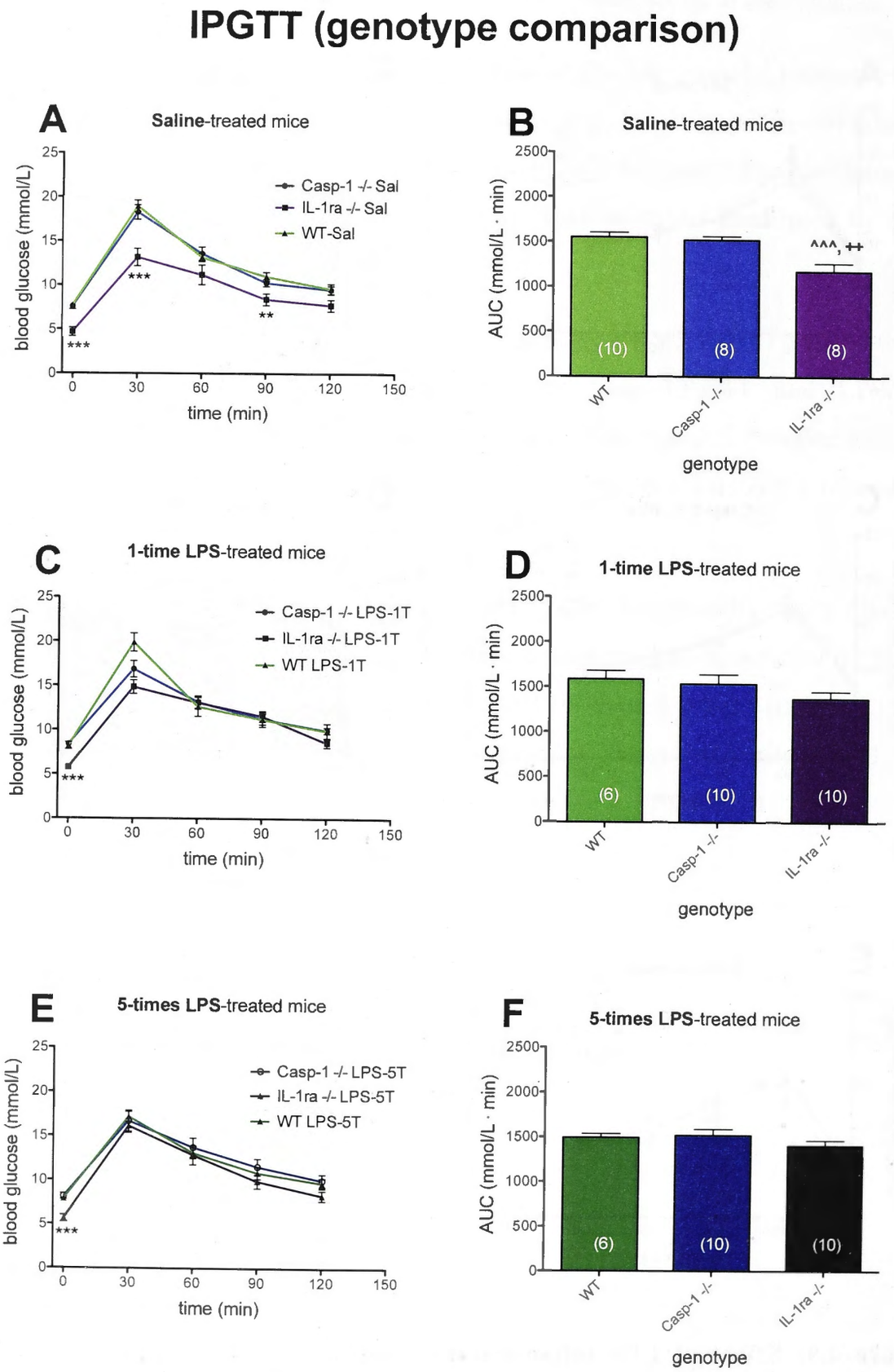


Figure 4.10: Effect of LPS inflammatory challenge on blood glucose level in IPGTT test: Mice were fasted for 6 hours and were injected (i.p.) with 2g/kg of glucose solution. Blood glucose level was measured at 0 min before injection and 30, 60, 90 and 120 min after the injection. *Comparison within each treatment*: Blood glucose levels of A)

Saline, C) LPS-1T and E) LPS-5T treated mice were measured in the three different genotypes and analyzed by one-way ANOVA (Tukey`s multiple comparison test). Glucose tolerance in B) Saline, D) LPS-1T and F) LPS-5T treated mice was analyzed by area under the curve (AUC) and compared within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. **$p<0.001$, ***$p<0.0001$ vs. WT and Casp-1-/-; ^^^$p<0.0001$ vs. WT, $^{++}p<0.001$ vs. Casp-1-/-.

At the end of the studies mice were euthanized and their plasma insulin level was measured by ELISA method in order to assess the long-term effects of LPS on functionality of glycemic control.

Figure 4.11 (A, B and C) represents the insulin level of three genotypes (WT, Casp-1-/- and IL-1ra-/-, respectively) in order to assess the effect of single and repeated LPS injections. As it may be observed in figure 4.11 (A, B and C), single and five LPS injections did not significantly alter the insulin level in any of the three genotypes.

Comparison among the genotypes indicated that Casp-1-/- mice tended to have elevated levels of insulin in comparison to other genotypes and in particular this was significantly different versus IL-1ra-/- mice (figure 4.11 D). Data gathered from three treatments and analyzed within each genotype, indicate that IL-1ra-/- mice had overall lower levels of insulin (figure 4.11 E). Casp-1-/- had higher level of insulin than IL-1ra-/- but it was not significant versus WT mice (figure 4.11 E).

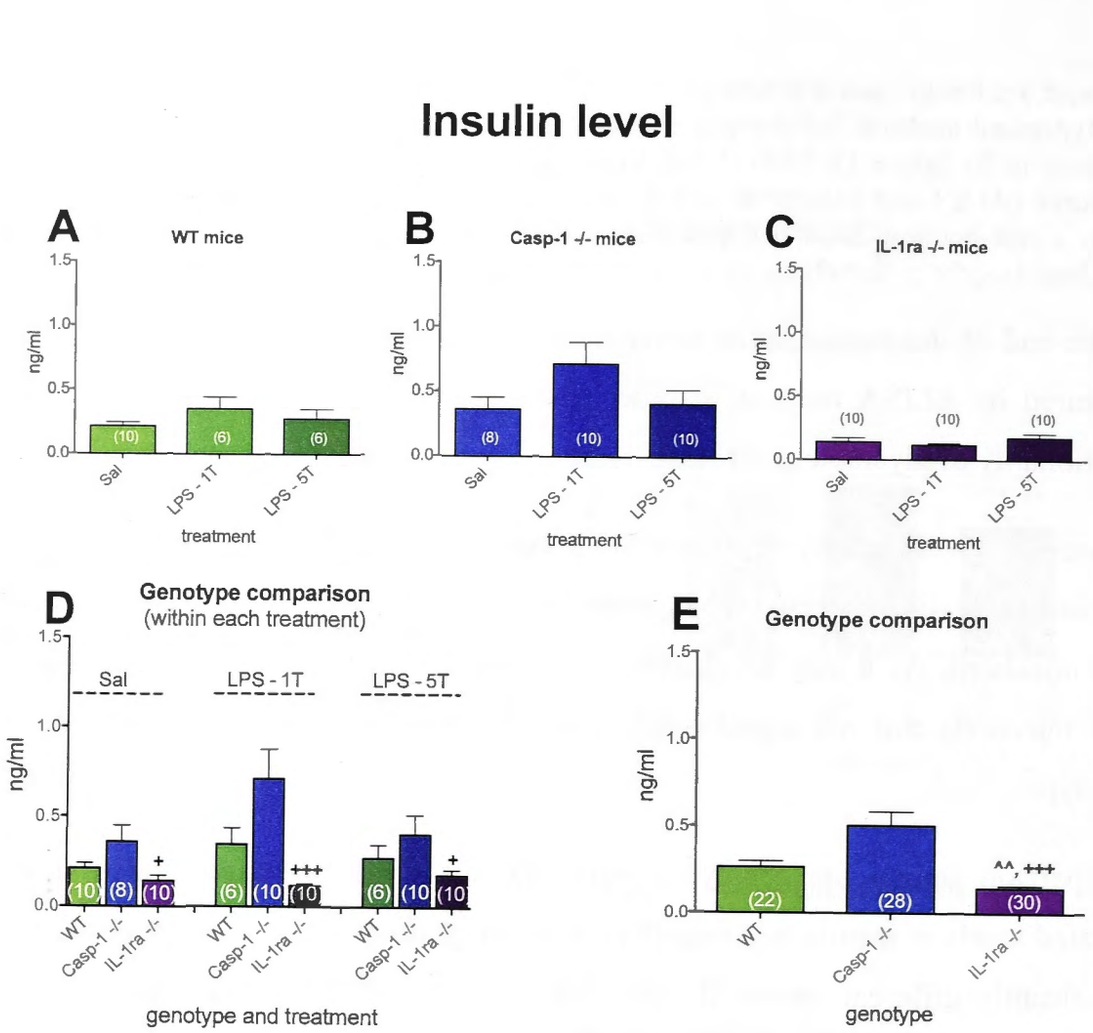


Figure 4.11: Effect of long-term LPS treatment on plasma level of insulin: The level of insulin in blood plasma was measured by ELISA kit and concentration was expressed in ng/ml. *Comparison within each genotype*: Levels of plasma insulin in A) WT, B) Casp-1-/- and C) IL-1ra-/- mice were compared by one-way ANOVA followed by Tukey`s multiple comparison test. *Comparison within each treatment*: D) Data collapsed from figures A), B) and C) were analyzed within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. E) Data summarized from figure D) were analyzed by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. ^^$p<0.001$ vs. WT; $^{+}p<0.05$, $^{+++}p<0.0001$ vs. Casp-1-/-.

Similarly, leptin levels were measured in blood plasma. The leptin level of IL-1ra-/- mice is not shown as it was extremely low or below detection. IL-1ra-/- mice tended to additionally lose 4-15% of their body weight during the time from DEXA scan and euthanasia, which might explain the extremely low levels of plasma leptin.

In figure 4.12 (A and B), the effect of single and repeated LPS injections was assessed in WT and Casp-1-/- mice, respectively. As it may be observed, leptin levels were not altered by LPS treatment in either genotype. Direct comparison between the two genotypes indicated that leptin levels were similar or tended to be elevated in WT mice when compared to Casp-1-/- mice (figure 4.12 C).

In figure 4.12 (D and E), leptin levels of two genotypes (WT and Casp-1-/- mice, respectively) were normalized by fat mass. The ratio between leptin and fat mass indicates that secretion of leptin in both WT and Casp-1-/- mice was not altered by LPS inflammatory challenge. Genotype comparison in saline and one time LPS-injected group indicates that leptin/fat mass ratio was significantly lower in Casp-1-/- mice than in WT mice (figure 4.12 F). These significant differences were not evident in the 5-times LPS-injected mice (figure 4.12 F). Overall, the data suggest that Casp-1-/- mice had lower secretion of leptin from adipose tissue.

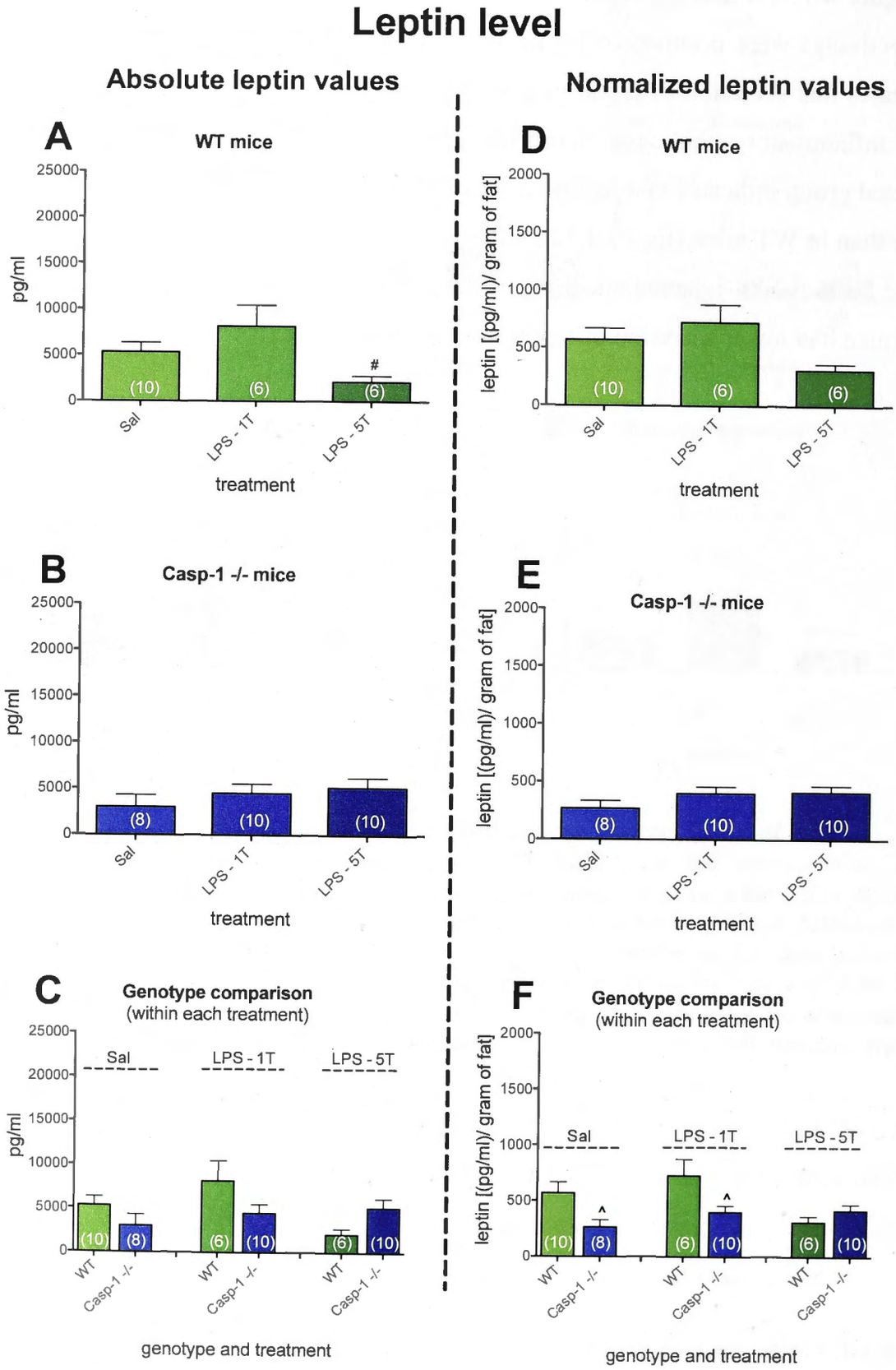


Figure 4.12: Leptin level measured in blood plasma: Level of plasma leptin was measured by ELISA kit and its concentration was expressed in pg/ml. *Comparison within each genotype*: Plasma leptin levels in A) WT and B) Casp-1-/- mice was compared by one-way ANOVA (Tukey's multiple comparison test). Leptin levels of D) WT and E) Casp-1-/- mice were normalized versus corresponding values of fat mass measured by DEXA scan and analyzed by one-way ANOVA followed by Tukey's post-hoc test. *Comparison within each treatment*: C) Leptin levels were compared between genotypes and within each treatment by

unpaired T-test. F) Leptin/fat mass ratio was compared between two genotypes within each treatment by unpaired T-test. Error bars represent standard errors. ^$p<0.05$ vs. WT, $^{\#}p<0.05$ vs. LPS-1T.

In order to assess the additional effects of long-term high-fat diet consumption anf LPS treatment on metabolic outcomes, IPGTT test, insulin and leptin levels tests were performed.

Figure 4.13 (A and C) presents the glucose level determined in IPGTT test of RD and HFD-fed mice in order to assess the effect of single and repeated LPS injections. As it may be observed, single or five LPS injections did not significantly alter glucose levels in RD and HFD groups. Moreover, the AUC analysis in the figure 4.13 (B and D) suggests that LPS treatment did not alter the glucose tolerance in either diet-fed mice.

With regards to the effect of diet, comparison within saline-treated groups indicated that HFD-fed mice had a higher baseline level of glucose and showed a significant increase in glucose intolerance at 60, 90 and 120 minutes after glucose injection (figure 4.14 A). Similarly, HFD-fed mice injected with either single or repeated LPS injections showed higher baseline glucose levels and lower glucose tolerance at various points in time (figure 4.14 C and E). Further analysis revealed that HFD-fed mice were glucose intolerant as shown in saline and single LPS-injected groups (figure 4.14 B and D), while there was a tendency for 5-times LPS-injected HFD-fed mice to be more glucose intolerant versus RD-fed mice (figure 4.14 F).

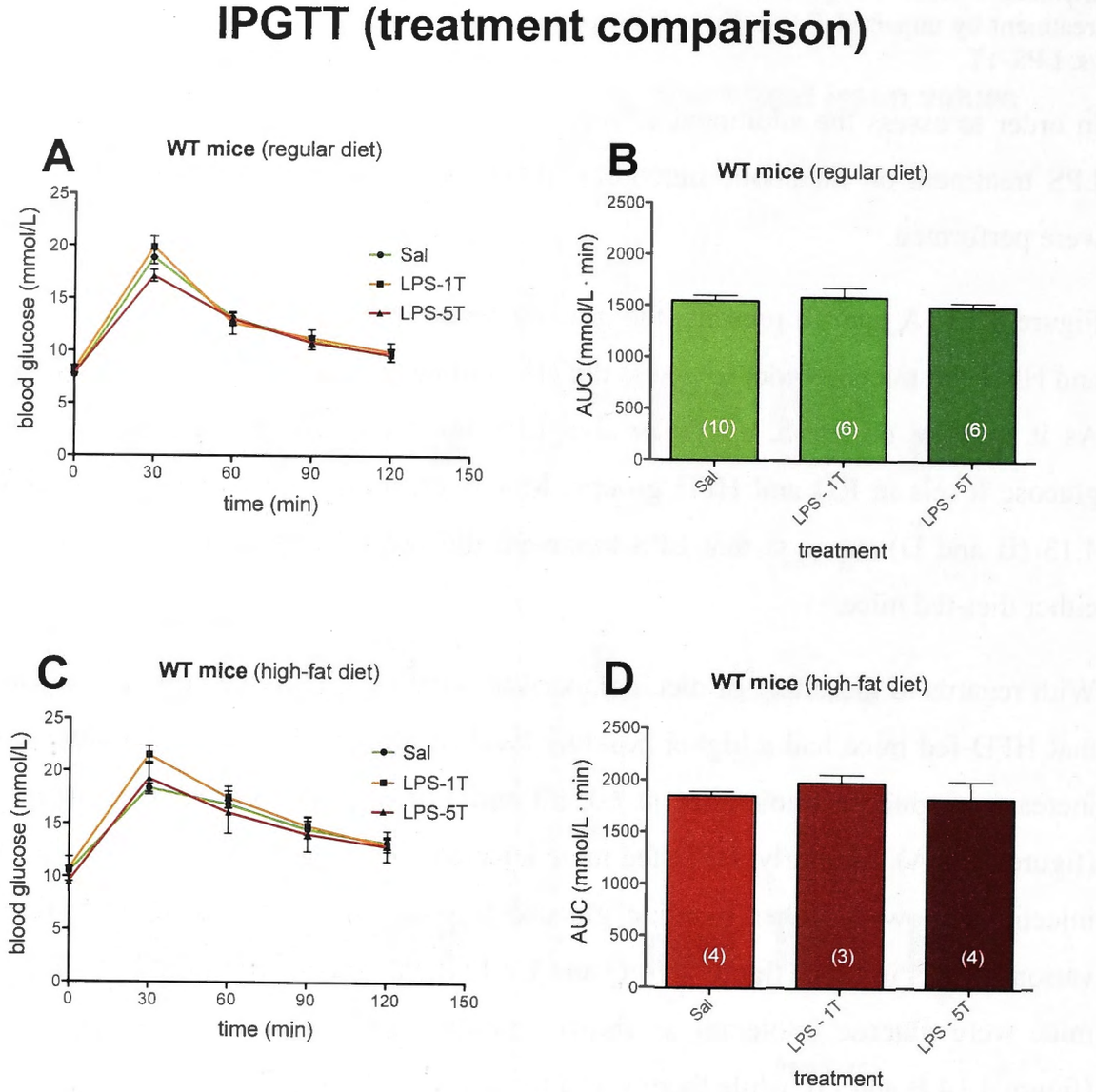


Figure 4.13: Effects of LPS inflammatory challenge and high-fat diet on blood glucose level in IPGTT test. Mice were fasted for 6 hours and were injected (i.p.) with 2g/kg of glucose solution. Blood glucose level was measured before injection (0 min) and 30, 60, 90 and 120 min after the glucose administration. *Comparison within each diet*: Blood glucose levels of A) RD and C) HFD-fed mice were analyzed by one-way ANOVA (Tukey`s multiple comparison test). Glucose tolerance in B) RD and D) HFD-fed mice was analyzed by AUC and differences were compared by one-way ANOVA (Tukey`s multiple comparison test). Error bars represent standard errors.

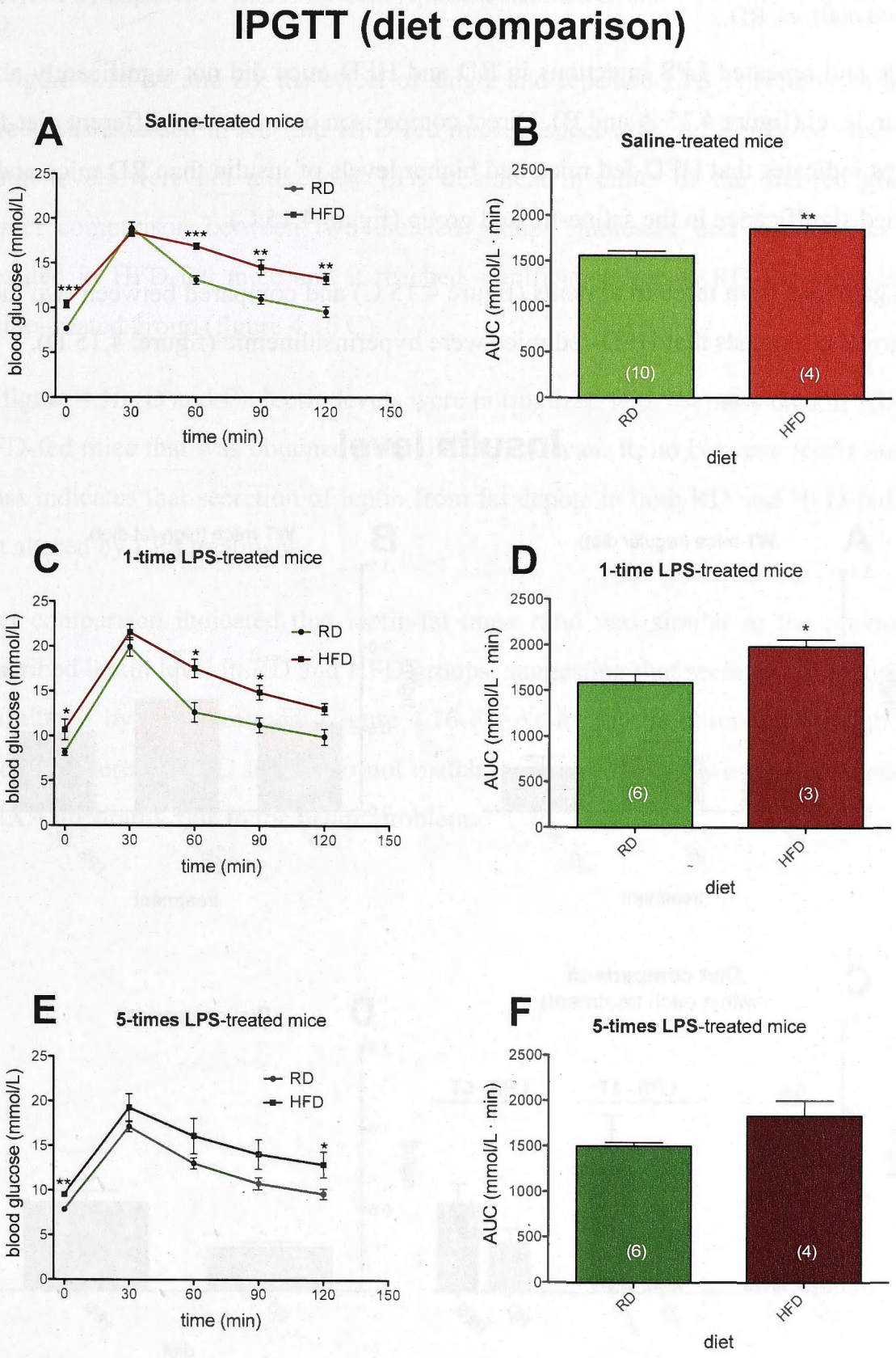


Figure 4.14: Effects of LPS inflammatory challenge and high-fat diet on blood glucose level in IPGTT test. Mice were fasted for 6 hours and were injected (i.p.) with 2g/kg of glucose solution. Blood glucose level was measured before injection (0 min) and 30, 60, 90 and 120 min after the glucose administration. *Comparison within each treatment*: Blood glucose levels in A) Saline, C) LPS-1T and E) LPS-5T treated mice were compared between two different diet-fed groups and analyzed by unpaired T-test. Glucose tolerance in B) Saline, D) LPS-1T and F) LPS-5T treated mice was analyzed by AUC and differences

were compared by unpaired T-test. Error bars represent standard errors. *p<0.05, **p<0.001, ***p<0.0001 vs. RD.

Single and repeated LPS injections in RD and HFD mice did not significantly alter insulin level (figure 4.15 A and B). Direct comparison between two different diet-fed groups indicates that HFD-fed mice had higher levels of insulin than RD mice and it reached significance in the saline-treated group (figure 4.15 C).

Data gathered from three treatments (figure 4.15 C) and compared between two diet-fed groups, suggests that HFD-fed mice were hyperinsulinemic (figure. 4.15 D).

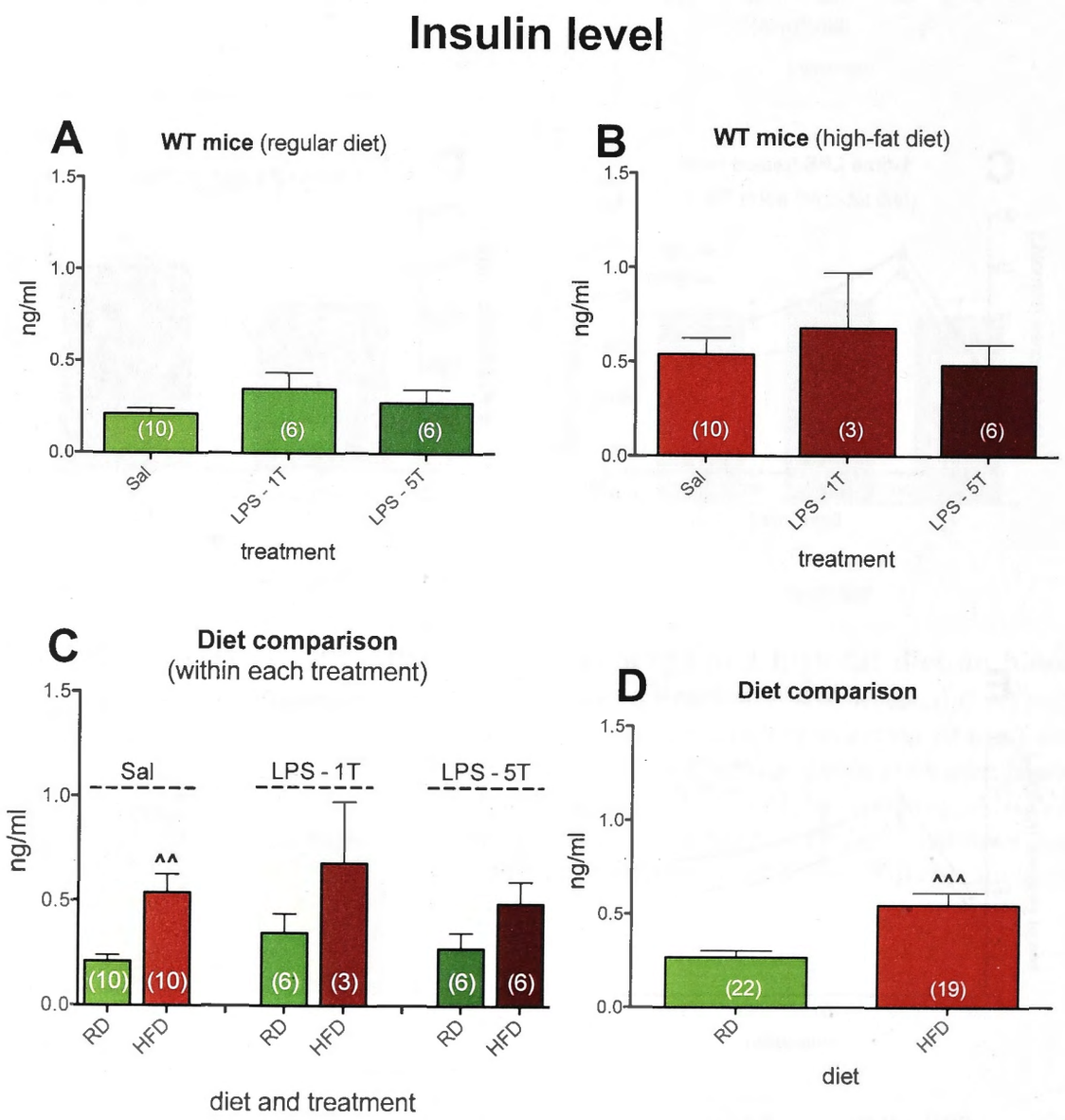


Figure 4.15: Effects of LPS inflammatory challenge and high-fat diet on insulin level in the blood: Level of insulin in the blood plasma was measured by ELISA kit and concentration was expressed in ng/ml. *Comparison within each diet*: Insulin levels of A) RD and B) HFD-fed mice were analyzed by one-way ANOVA (Tukey's multiple comparison test). *Comparison within each treatment*: C) Data collapsed from figures A) and B) were analyzed within each treatment by unpaired T-test. D) Data summarized from figure C) were

analyzed by unpaired T-test. Error bars represent standard errors. ^^p<0.01, ^^^p<0.001 vs. RD.

In figure 4.16 (A and B), the effect of single and repeated LPS injections on leptin levels was assessed in RD and HFD-fed mice, respectively. As it may be observed, leptin levels were not altered by LPS treatment in either of the diet-fed groups. Direct comparison between two diet-fed groups, indicates that leptin level was elevated in HFD-fed mice and it reached significance versus RD-fed mice in the saline-treated group (figure 4.16 C).

In figure 4.16 (D and E), leptin levels were normalized with fat mass data of RD and HFD-fed mice that was obtained from the DEXA scan. Ratio between leptin and fat mass indicates that secretion of leptin from fat depots in both RD and HFD-fed was not altered by LPS treatment.

Diet comparison indicated that leptin/fat mass ratio was similar to the previously described leptin level in RD and HFD groups, suggesting that secretion of leptin was not altered by LPS treatment (figure 4.16 F). As it may be observed, a number of mice in figure 4.16 (C and F) do not match, as some of mice were not scanned by DEXA apparatus, due to the health problem.

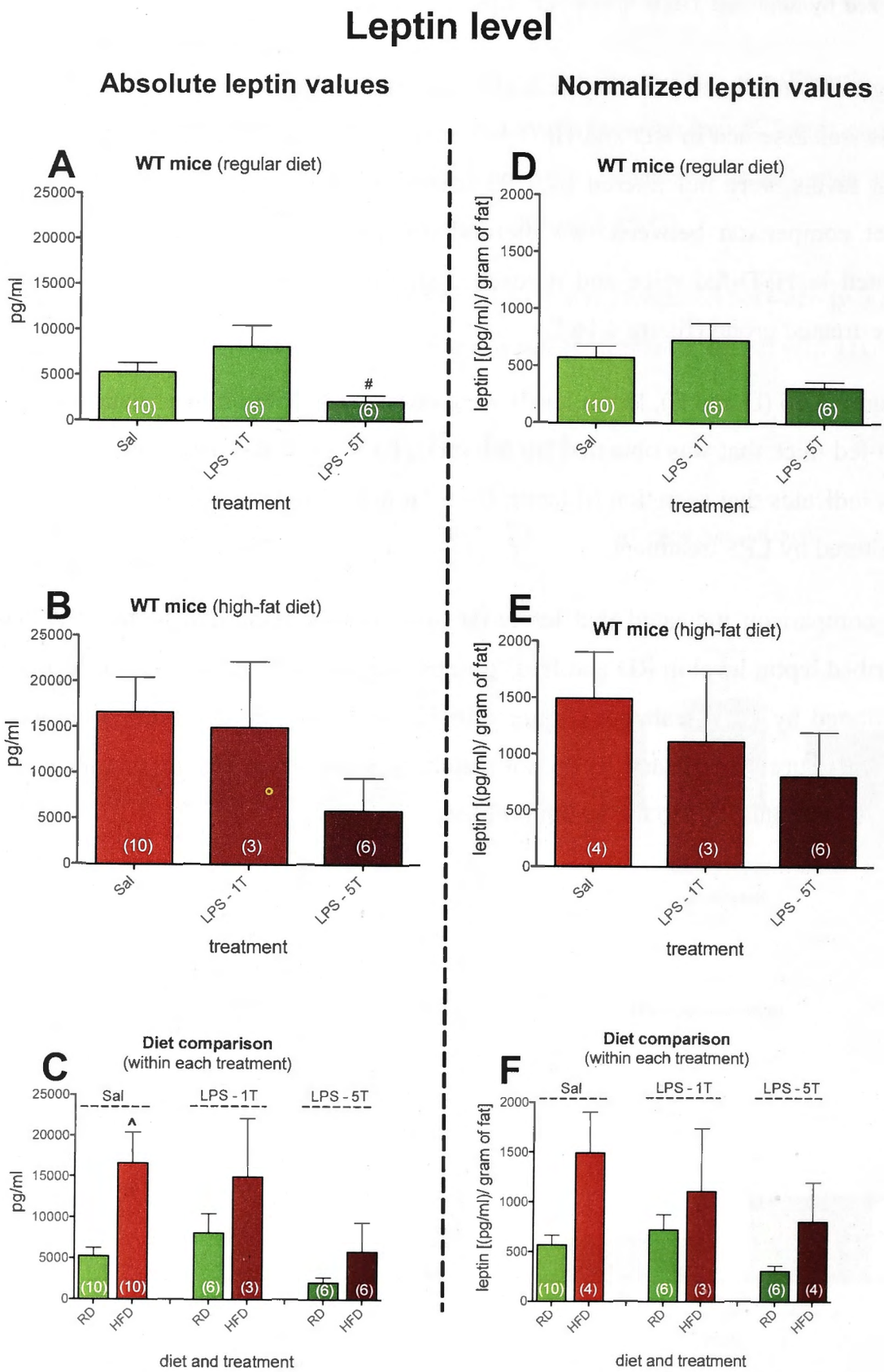


Figure 4.16: Effects of LPS inflammatory challenge and high-fat diet on leptin blood level: *Comparison within each diet*: Levels of plasma leptin of A) RD and B) HFD-fed mice were compared by one-way ANOVA (Tukey`s multiple comparison test). Leptin levels of D) RD and E) HFD-fed mice were normalized versus corresponding values of fat mass tissue measured by DEXA scan and analyzed by one-way ANOVA followed by Tukey`s post-hoc test. *Comparison within each treatment*: C) Leptin levels were compared between RD and HFD-fed mice by unpaired T-test. F) Leptin/fat mass ratio was compared

between RD and HFD-fed mice by unpaired T-test. Error bars represent standard errors. #p<0.05 vs. LPS-1T, ^p<0.05 vs. RD.

Since HFD-fed mice did not significantly gain weight despite increased food intake, a new set of mice was used to ascertain if frequent behavioral tests in addition to the five monthly (i.p.) injections could have interfered with body weight gain. These frequent interventions could have induced mild-chronic stress affecting weight gain in HFD-fed mice. Therefore, a new set of mice that were exposed to a minimum number of behavioral tests, but not injected, was used to ascertain the possible effect of frequent interventions (i.p. injections and repeated behavioral tests) on metabolic outcomes.

For this purpose, the following two groups of mice were studied:

- Non -injected WT mice fed a regular diet (RD-NI) (n=10)

- Non- injected WT mice fed a high-fat diet (HFD-NI) (n=10)

Mice were kept under the same environmental conditions that were previously described in chapter 2. The new set of mice (RD-NI and HFD-NI) was subjected to the metabolic tests as it was described for the previous set of experimental mice. Then, they were compared with the saline-injected RD and HFD-fed mice.

Brief methodology:

1) Metabolic studies: Mice were subjected to IPGTT test and body composition was determined by DEXA scan at the end of 13 months. At the end of 15 months of *in vivo* studies, plasma levels of leptin and insulin were ascertained.

Metabolic studies:

Figure 4.17 (A and C) shows the results of the IPGTT test conducted in saline-injected and non-injected RD and HFD-fed mice, respectively. As it may be observed both groups of HFD-fed mice were shown to be less glucose tolerant at 60, 90 and 120 min after the glucose injection (figure 4.17 A and C). In particular, HFD-NI mice showed first signs of glucose intolerance just 30 min post-injection (figure 4.17 C). Moreover, the AUC analysis in figure 4.17 (B and D) suggests that both HFD and HFD-NI mice were glucose intolerant.

With regards to the effect of treatment, the comparison between RD and RD-NI mice showed no difference in glucose tolerance between these two groups (figure 4.17 E and F). In the case of high-fat diet-fed mice, there was a tendency for HFD-NI mice to be less glucose tolerant than HFD-fed mice (figure 4.17 G). HFD-NI mice reached significance versus HFD-fed mice 30 min after glucose injection (figure 4.17 G). The AUC analysis has shown that HFD-NI mice displayed a trend to be more glucose intolerant than HFD-fed mice (figure 4.17 H).

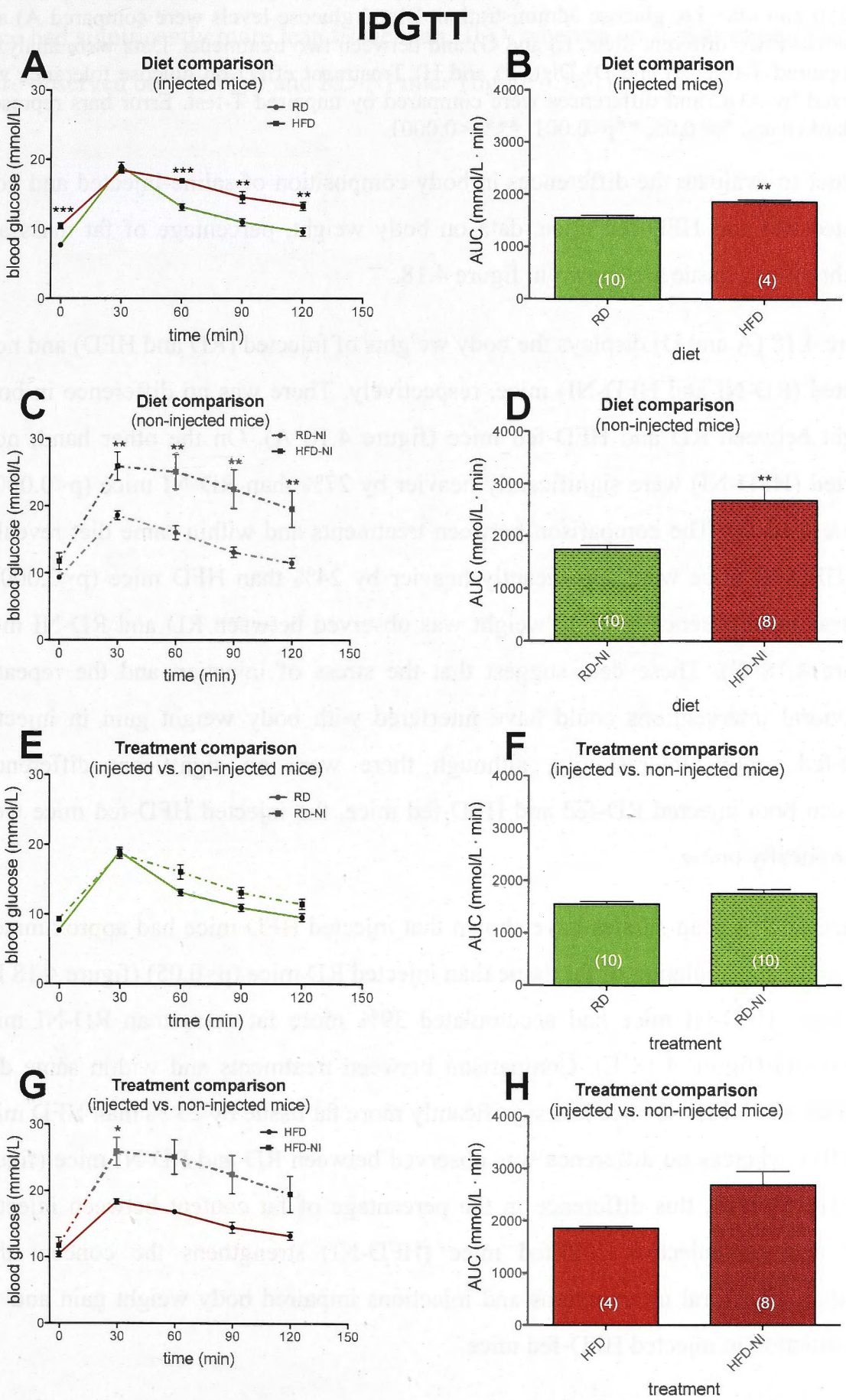


Figure 4.17: IPGTT test in saline-injected and non-injected RD and HFD-fed mice: Mice were fasted for 6 hours. Thereafter, they were injected (i.p.) with 2g/kg of

glucose solution. Blood glucose level was measured before injection (0 min) and 30, 60, 90 and 120 min after i.p. glucose administration. Blood glucose levels were compared A) and C) between two different diets; E) and G) and between two treatments. Data were analyzed by unpaired T-test. B) and D) Diet; F) and H) Treatment effect on glucose tolerance was analyzed by AUC and differences were compared by unpaired T-test. Error bars represent standard errors. *$p<0.05$, **$p<0.001$, ***$p<0.0001$.

In order to evaluate the differences in body composition of saline-injected and non-injected RD and HFD-fed mice, data on body weight, percentage of fat mass and weight of lean tissue are shown in figure 4.18.

Figure 4.18 (A and D) displays the body weights of injected (RD and HFD) and non-injected (RD-NI and HFD-NI) mice, respectively. There was no difference in body weight between RD and HFD-fed mice (figure 4.18 A). On the other hand, non-injected (HFD-NI) were significantly heavier by 27% than RD-NI mice ($p<0.0001$) (figure 4.18 D). The comparison between treatments and within same diet revealed that HFD-NI mice were significantly heavier by 24% than HFD mice ($p<0.0001$), whereas no difference in body weight was observed between RD and RD-NI mice (figure 4.18 G). These data suggest that the stress of injection and the repeated behavioral interventions could have interfered with body weight gain in injected HFD-fed group. Interestingly, although there were no significant differences between both injected RD-fed and HFD-fed mice, the injected HFD-fed mice were metabolically obese.

In fact, DEXA scan studies have shown that injected HFD mice had approximately 27% more accumulation of fat tissue than injected RD mice ($p<0.05$) (figure 4.18 B). Similarly, HFD-NI mice had accumulated 39% more fat mass than RD-NI mice ($p<0.0001$) (figure 4.18 E). Comparison between treatments and within same diet revealed that HFD-NI mice had significantly more fat tissue by 25 % than HFD mice ($p<0.05$), whereas no difference was observed between RD and RD-NI mice (figure 4.18 H). Overall, this difference in the percentage of fat content between injected HFD and non-injected HFD-fed mice (HFD-NI) strengthens the concept that repeated behavioral interventions and injections impaired body weight gain and fat accumulation in injected HFD-fed mice.

Analysis of lean tissue shows that injected HFD-fed mice had lower weight of lean tissue than RD mice (figure 4.18 C), whereas no significant differences were observed between the weight of lean tissue of RD-NI and HFD-NI mice (figure 4.18

F). Comparison between treatments and within the same diet revealed that HFD-NI mice had significantly more lean tissue than HFD, whereas no significant differences were observed between RD and RD-NI mice (figure 4.18 I).

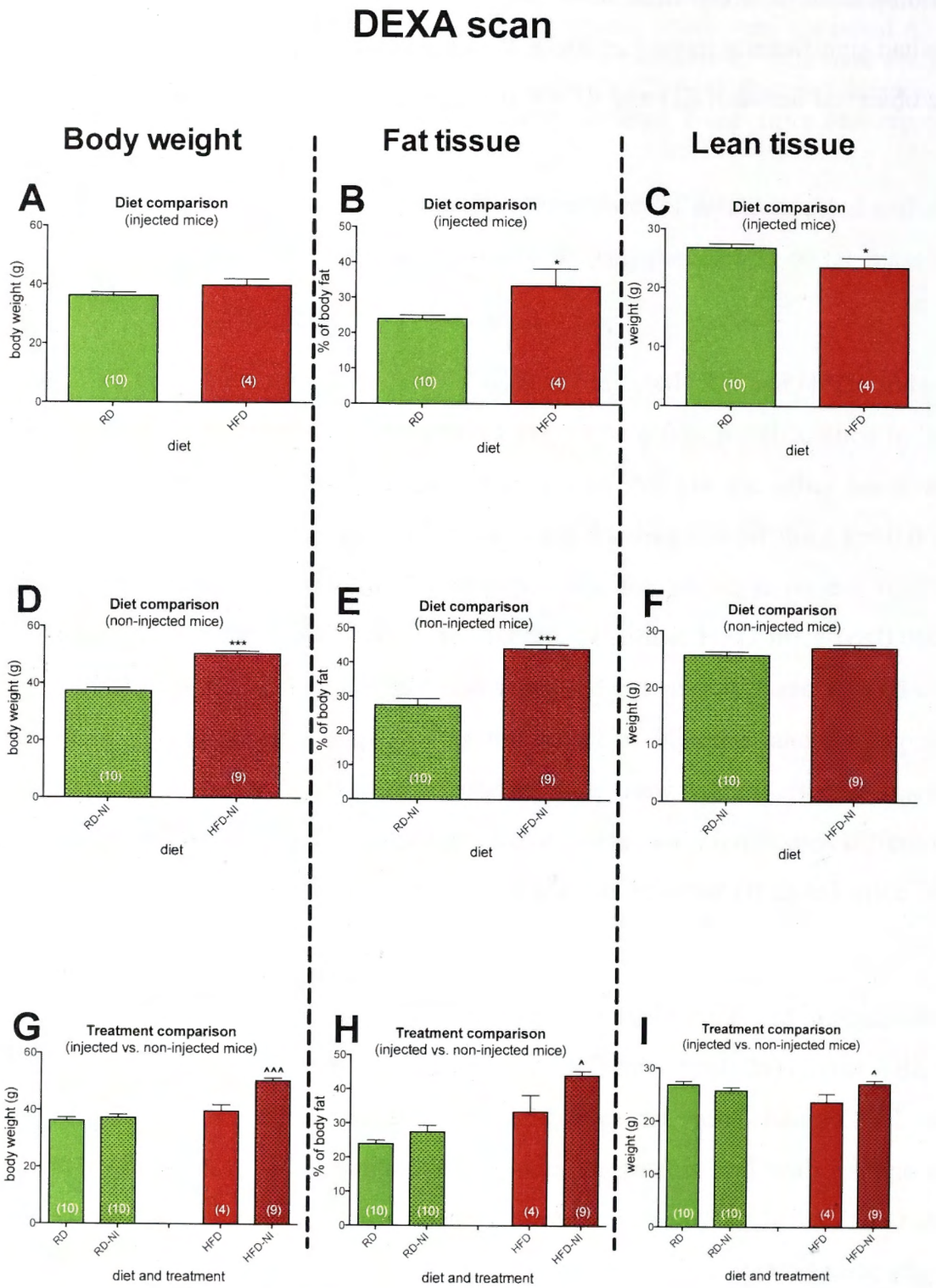


Figure 4.18: DEXA scan of injected and non-injected RD and HFD fed mice: *Comparison between diets*: Data on A) and D) Body weight; B) and E) Percentages of body fat mass and C) and F) weight of lean tissue within each diet were analyzed by unpaired-T test. *Comparison between treatments*: Data on G) Body weight, H) percentages of body fat mass and I) weight of lean tissue were compared between injected and non-injected RD and HFD-fed mice within each diet and analyzed by unpaired T-test. Error bars represent standard errors. *$p<0.05$, ***$p<0.0001$; ^$p<0.05$, ^^^$p<0.0001$ vs. HFD.

At the end of the *in vivo* studies, levels of insulin and leptin were measured in plasma. Levels of insulin were elevated in both HFD and HFD-NI mice (figure 4.19

A and B). The comparison within the same diet indicated that RD-NI had higher plasma insulin than RD ($p<0.01$) (figure 4.19 C). Similarly, insulin was higher in HFD-NI than in HFD-fed mice ($p<0.01$) (figure 4.19 C).

Plasma leptin was elevated in both HFD and HFD-NI mice in comparison to their control groups ($p<0.05$ and $p<0.001$, respectively) (figure 4.19 D and E). The comparison within each diet showed that RD-NI had elevated, but not significantly, plasma leptin levels than injected RD-fed mice, whereas HFD-NI mice had significantly more plasma leptin levels than that of HFD-fed mice ($p<0.05$) (figure 4.19 F).

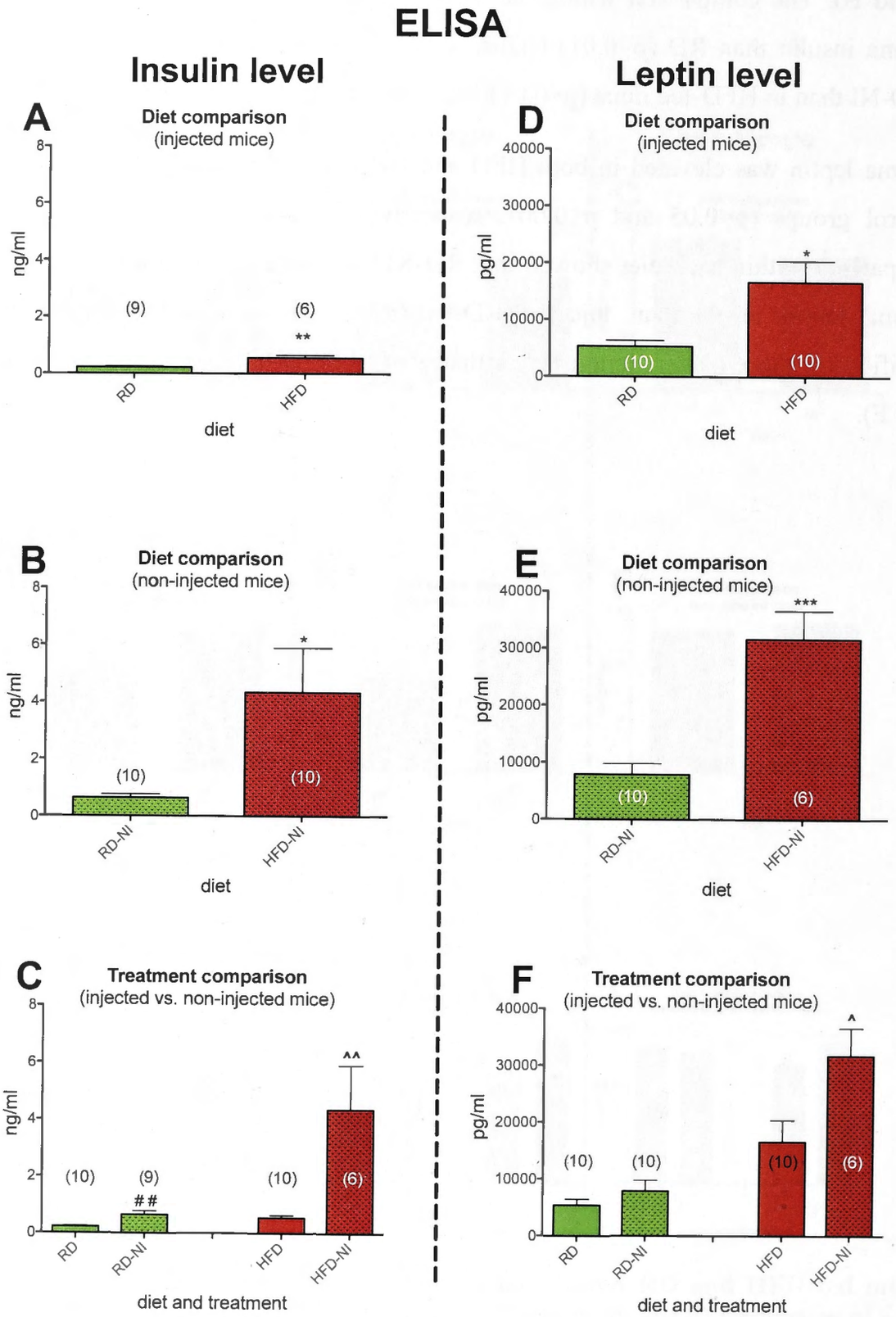


Figure 4.19: Insulin and leptin levels in injected and non-injected RD and HFD fed mice: *Comparison between diets*: Data on A) and B) Plasma insulin; D) and E) Leptin levels were compared by unpaired T-test. *Comparison between treatments*: Data on C) Insulin and F) leptin levels were compared by unpaired T-test. Error bars represent standard errors. *p<0.05, **p<0.01, ***p<0.0001; ##p<0.01 vs. RD; ^p<0.05, ^^p<0.01 vs. HFD.

Overall, data obtained from non-injected RD and HFD-fed mice suggest that saline injections could have produced mild-chronic stress that disabled mice fed a high-fat

diet from gaining significantly more weight than their control group. Non-injected HFD-fed mice gained significantly more weight, had more accumulated fat tissue and higher levels of leptin and insulin than injected HFD-fed mice. In spite of these significant differences, the injected HFD-fed mice still showed to be metabolically obese since they displayed glucose intolerance and had higher plasma levels of insulin and leptin in comparison to their control group (injected RD-fed mice).

4.5 Discussion

Metabolic perturbations such as type-2 diabetes, insulin resistance and obesity have been implicated in the etiology of several neurodegenerative disorders [556, 557]. In a recent study it has been shown that cognitive and motor functions in patients suffering from neurodegenerative disorders were improved when metabolic homeostasis was restored with acquired therapies [558]. Diabetes has been shown to reduce the content of dopamine within the nigrostriatal system [10]. Low levels of insulin in streptozotocin-induced diabetic rats were associated with decreased mRNA expression of DAT and tyrosine-hydroxylase in SNpc [559]. In parallel with the previous statement, increased phosphorylation of insulin receptor substrate-2, a marker of insulin resistance was observed in striatum of dopamine depleted 6-OHDA-mouse model of Parkinson`s disease [560]. Correlation between type-2 diabetes and Parkinson`s disease was reported in more than 50% of cases [560]. Insulin injection into mice brains increases the activity of DAT in substantia nigra [561], but chronic hyperinsulinemia has been shown to reduce the expression of insulin receptors in the BBB, which leads to central insulin resistance [562]. Chronic high-fat diet consumption in mice induced insulin resistance and decreased the release and clearance of dopamine from TH-neurons [12]. It has been shown that in *ob/ob* mice that are genetically prone to obesity and diabetes, also have low expression of TH enzyme in dopamine neurons [531]. Therefore the general aim of this chapter was to ascertain the long-term effect of systemic LPS inflammatory challenge and high-fat diet consumption on the occurrence and progression of metabolic disorders that may lead to impaired dopaminenergic-mediated neurotransmission and contribute to depletion of dopamine neurons.

AIM 1: Long-term effect of systemic LPS challenge on metabolic outcomes, exerted through the IL-1 pathway.

The IL-1 mediated signaling is not only implicated in the inflammatory process, it also plays a major role in general energy homeostasis. In this section, the physiological and pathological role of IL-1 was examined on feeding behavior, body composition, glucose tolerance and plasma levels of insulin and leptin. With regards to the physiological role of IL-1, major differences in metabolic functions were observed among the three genotypes (WT, Casp-1-/- and IL-1ra-/-):

- Casp-1-/- mice displayed increased food intake that was not followed by an increase of body weight. Assessment of body composition confirmed that increased food intake in Casp-1-/- mice did not result in increased accumulation of fat tissue and no change was observed in weight of lean tissue. Despite similar body length to WT mice, Casp-1-/- mice had increased BMC and BMD. Casp-1-/- mice were not glucose intolerant but had increased plasma levels of insulin that was accompanied by a decrease of plasma leptin.
- IL-1ra-/- mice showed increased food intake, growth retardation and decreased accumulation of body fat mass. Decreased longitudinal growth was accompanied by reduced BMC, but possible over-stimulation of IL-1 signaling in IL-1ra-/- mice resulted in diminished BMD. IL-1ra-/- mice were more insulin sensitive and showed reduced plasma insulin. Decreased fat mass was accompanied by low levels of leptin that in most cases was below the sensitivity of the assay.

The long-term effect of LPS inflammatory challenge, has altered some of the metabolic features that were described above:

- Repeated LPS inflammatory challenge increased food intake in WT mice when adjusted to the body weight. Although single and repeated LPS injections tended to reduce the body weight gain, DEXA scan showed that body fat mass was only reduced with repeated LPS injections. Although, longitudinal growth was not affected by LPS inflammatory challenge, BMD and BMC were reduced in the 5-times LPS-injected mice. In WT mice, systemic LPS treatment neither reduced glucose tolerance nor did it alter plasma levels of leptin and insulin.

- Food consumption of Casp-1-/- mice was not altered by LPS administration. There was no reduction in body weight gain, and as it was later confirmed by DEXA scan, body fat mass and lean tissue did not decrease. Although there was no difference in body length, single and repeated LPS injections decreased BMD and BMC. However, systemic LPS inflammatory challenge neither induced glucose intolerance nor it did alter the insulin and leptin level.
- Repeated LPS inflammatory challenge reduced the food intake in IL-1ra-/- mice but without affecting their weight gain, the body fat mass and lean tissue. Similarly, neither longitudinal growth nor BMD and BMC were reduced. However, single and repeated LPS injections reduced the insulin sensitivity in IL-1ra-/- mice without affecting insulin and leptin levels.

The role of IL-1 and LPS inflammatory challenge on the modulation of the feeding process was ascertained in groups of WT, Casp-1-/- and IL-1ra-/- mice. Single and repeated LPS injections did not alter food intake in the three genotypes. When food intake was adjusted to body weight then it was observed that systemic LPS inflammatory challenge increased the food intake in 5-times LPS-injected WT mice, decreased food intake in IL-1ra-/- mice and no LPS effect was observed in Casp-1-/- mice. The reduced feeding in IL-1ra-/- mice by repeated LPS injections is in agreement with previous study where exogenous administration of IL-1 in rats reduced the feeding through the mechanism that is regulated by central melanocortin- 3/ 4 receptors (MC3/4 R`s) [563]. Similarly, intraperitoneal administration of LPS in mice induced anorexia via IL-1 mediated central action in the hypothalamus [564]. The genotype comparison indicated that food intake of Casp-1-/- mice was elevated in saline-treated group. Food intake was previously shown to be elevated in high-fat diet-fed Casp-1-/- mice [565]. Similarly, food intake was higher in IL-1ra-/- mice. It is suggested that under physiological conditions, over-activation of IL-1 signaling might be too weak to suppress the feeding process and only a large excess of IL-1 can suppress the appetite [566].

Systemic LPS inflammatory challenge did not decrease the body weight gain in any of the three genotypes. In the current study, single and repeated LPS injections did induce the body weight loss in the first week after the injection (data not shown) but mice managed the recover their weight gain two to three weeks post-injection. As

shown previously, short-term LPS injection reduced the body weight gain in pre-weanling rats [567]. The initial body weight measurements were taken when mice of all three genotypes (WT, Casp-1-/- and IL-1ra-/-) reached 40 days of age. The body weight data analysis indicated that Casp-1-/- mice were heavier, whereas IL-1ra-/- mice were the leanest. It has been observed earlier that IL-1-ra-/- mice suffered from growth retardation after the weaning and consequently had lower body weight [378]. Although, initial body weight of Casp-1-/- mice was higher, in other study it was shown that 6-months old caspase-1 deficient mice had similar body weight as WT mice [568]. Similarly, body weight data taken after 12 months indicated that body weights of WT and Casp-1-/- mice were similar. As it was mentioned above, Casp-1-/- mice had increased food intake but their final body weight was similar to WT mice. It has been reported that caspase-1 deficient mice have higher energy expenditure [565]. As it was described in chapter 3, the locomotor activity of these mice was significantly higher, which also supports the idea of non-significant weight gain in Casp-1-/- mice. It has been shown that caspase-1 deficient mice fed a high-fat diet had increased food intake and lower accumulation of white adipose tissue than WT mice on the same diet [565, 569]. Moreover it has been shown that high-fat diet-fed Casp-1-/- mice had smaller adipocytes but their differentiation was not impaired in absence of caspase-1 [568]. Similar study showed that decreased accumulation of fat in Casp-1-/- mice is due to decreased intestinal absorption of triglyceride and reduced production of hepatic very low-density lipoprotein-triglycerides [569]. Cumulatively, these observations suggest that increased energy expenditure and decreased triglyceride accumulation in fat depots of Casp-1-/- mice could have resulted in non-significant weight gain despite higher food intake.

Body composition suggested that repeated LPS treatment decreased the body fat accumulation in WT mice. Results from *in vitro* studies indicated that IL-1 inhibits the synthesis of proteins involved in the transport of fatty acids to adipose tissues [570]. This might imply that ongoing peripheral inflammation in 5-times LPS-injected WT mice might have reduced the accumulation of fat tissue and subsequently this could account for increased food intake. On the other hand, neither single nor repeated LPS inflammatory challenges decreased the fat accumulation in Casp-1-/- and IL-1ra-/- mice. Since genotype comparison indicated that IL-1ra-/- mice had the least percentage of fat mass, it might be suggested that the amount of

accumulated fat in IL-1ra-/- mice was so low that it could not be further decreased by repeated LPS interventions. Decreased accumulation of adipose tissue combined with increased food intake as a ratio of body weight has been associated with increased energy expenditure and defective adipogenesis in IL-1ra-/- mice [571]. Deficiency in IL-1ra led to altered morphology of white adipose tissue, which was associated with impaired differentiation of adipocytes [571]. In particular it was observed that lack of IL-1ra in mice was followed by decreased expression of transcription factors involved in adipocyte differentiation [571].

As it was described previously, systemic administration of cytokines such as TNF-α may stimulate fat degeneration [572, 573]; in a similar manner it has been shown that cytokines such as TNF-α and IL-1 are involved in muscle atrophy [574, 575]. DEXA scans have shown that long-term effects of single and repeated LPS inflammatory challenges did not induce muscle wasting in any of the three genotypes.

Initial body length comparison among genotypes indicated that IL-1ra-/- mice were the smallest, which is due to growth retardation [378], whereas Casp-1-/- mice were the longest. At the end of 13 months Casp-1-/- and WT mice had similar body lengths, whereas IL-1ra-/- mice did not catch up with the other two genotypes. Assessment of bone composition suggested that repeated LPS injections reduced the BMD and BMC in WT mice. Proinflammatory cytokines contribute to bone resorption by stimulating the activity of osteoclasts, as it was shown in cystic fibrosis, an example of systemic inflammation [576]. Similar reduction in BMD and BMC was observed in Casp-1-/- mice that were induced with single and repeated LPS injections, whereas no LPS effect was shown in IL-1ra-/- mice. In regard to the LPS effect on bone resorption observed in Casp-1-/- mice, it has been shown that caspase-1 gene deficient mice are not protected against induced inflammatory arthritis as it was demonstrated that neutrophil serine proteinase-3 contributes to the production of bioactive IL-1β [577]. Genotype comparison indicated that Casp-1-/- mice had higher BMD and BMC, whereas IL-1ra-/- had the lowest in saline-treated groups. It was previously observed that Casp-1-/- mice had higher BMC than WT mice [569]. Similarly, IL-1 knockout mice (IL-1α -/-, IL-1β -/- and IL-1α/β -/- mice) exhibited an increase in bone mass that was followed by a decrease in the number of

osteoclasts [578]. Conversely, an excess of IL-1, such as that found in IL-1ra-/- mice, was associated with development of arthritis and bone destruction [579]. Five LPS injections reduced the BMD and BMC in WT and Casp-1-/- to the level that was similar to IL-1ra-/- mice.

Measurements of glucose tolerance indicated that IL-1ra-/- mice were more glucose tolerant than Casp-1-/- and WT mice in saline-treated groups. As it was described before, IL-1ra-/- mice are reported to have increased insulin sensitivity [566]. On the other hand, this glucose tolerance in IL-1ra-/- mice was diminished with single and repeated LPS injections.

Single and repeated LPS injections did not impair insulin or leptin secretion in any of the three genotypes. One study has shown that IL-1 could be antidiabetic [580]. Conversely, other study demonstrated that IL-1 induced the death of pancreatic β-cells [581]. IL-1ra-/- mice are reported to have impaired insulin secretion that results in hypoinsulinemia [566]. As insulin has an important role in pre and post-natal growth [582], hypoinsulinemia in IL-1ra-/- mice could explain postnatal growth retardation in these mice. Normal prenatal growth in IL-1ra-/- mice could be attributed to normal *in utero* levels of insulin and pre-weaning supply of insulin from milk could account for normal body weight at the weaning. Similarly, leptin levels in IL-1ra-/- mice were extremely low (data not presented), which was associated with a very low percentage of fat by the end of the study. As it was previously reported, circulating leptin levels were reduced by 6-fold in IL-1ra-/- mice [571]. On the other hand, the secretion of insulin in Casp-1-/- mice was increased. The increase in plasma insulin in Casp-1-/- mice could have resulted from decreased secretion of leptin from adipose tissue. It has been shown that acute administration (i.p.) of IL-1 in mice increases the serum level of leptin and mRNA expression [583], which could suggest that a lack of IL-1 signaling in Casp-1-/- mice could have impaired the secretion of leptin from adipose tissue. Leptin levels in WT mice tended to be lower with repeated LPS injections, since their fat accumulation was lower.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on metabolic outcomes.

In this section the effects of high-fat diet and LPS inflammatory challenge were examined on feeding behavior, body composition, glucose tolerance and plasma levels of insulin and leptin.

Major differences in metabolic functions have been observed between HFD and RD-fed mice, whereas LPS treatment did not exacerbate the metabolic outcomes in either diet-fed group of mice:

- High-fat diet consumption increased the food intake, which was not further altered by systemic LPS treatment. On the other hand, repeated LPS injections increased food intake as a ratio of body weight in RD-fed mice. Despite increased food intake, HFD-fed mice had similar body weight to RD-fed mice. In both diet-fed groups, LPS inflammatory challenge decreased, but not significantly, body weight. Decrease in body weight in both RD and HFD-fed mice with repeated LPS injections was further verified with DEXA scan as the accumulation of body fat was lower in RD mice, but not significantly, compared to HFD-fed mice. A minor reduction in lean tissue was observed in HFD-fed mice. Long-term effects of systemic LPS did not alter the longitudinal growth, but repeated inflammatory challenges decreased the BMC and BMD in RD-fed mice; moreover there was a trend for these parameters to be increased in HFD-fed mice. HFD-fed mice showed to be glucose intolerant and had higher plasma levels of insulin and leptin. No additional effects of LPS were observed on metabolic parameters (glucose tolerance, leptin and insulin level) in either diet-fed groups.

Repeated LPS inflammatory challenges increased the food intake in RD mice. As it was described above, an increase in food intake in RD mice after five LPS injections could have been attributed to lower accumulation of fat tissue and lower, but not significantly so, plasma level of leptin. On the other hand, a high-fat diet did not exacerbate the effect of LPS treatment on the feeding process. Average food consumption was increased in HFD-fed mice and also remained higher relative to body weight. It has been already described that chronic high-fat diet consumption alters the homeostatic regulation of energy balance and leads to hyperphagia [584, 585].

There was a slight tendency for repeated LPS injections to reduce body weight in RD and HFD-fed mice. Surprisingly, despite higher food intake, HFD-fed mice had similar body weight to RD-fed mice and it remained similar with single and repeated LPS injections. We hypothesize that frequent interventions such as injections, body length measurements and behavioral assessments could have resulted in reduced weight gain in HFD-fed mice. As it was described in the current study (figure 4.18), a new batch of non-injected and high-fat-diet fed mice (HFD-NI) mice gained significantly more weight in comparison to saline-injected HFD-fed mice. These additional data suggest that frequent behavioral interventions coupled with (i.p.) injections might have affected weight gain.

DEXA scans have shown that five LPS injections reduced percentage of accumulated fat tissue in RD-fed mice and similarly there was a trend in HFD-fed mice to have decreased percentage of fat. Although the food intake in five times LPS-injected RD and HFD-fed mice was higher or similar, respectively, reduction in fat accumulation could have been related to IL-1 signaling. As it was mentioned before, IL-1 inhibits he synthesis of proteins involved in the transport of fatty acid to adipose tissue in *in vitro* study [570]. It could be suggested that repeated LPS injections had a long-term negative effect on fat storage. Comparison between RD and HFD-fed mice has shown that HFD-fed mice had tendencies to have more accumulated fat tissue, which was expected from the higher food intake.

Single and repeated LPS inflammatory challenge did not induce muscle atrophy in either RD or HFD-fed mice. It has been proposed that dyslipidaemia in high-fat diet-fed rats could have resulted in activation of proinflammatory cascade, which could have led to atrophy of skeletal muscles [586]. In the current study, only the significant increase in the percentage of body fat of saline-treated HFD-fed mice might have been related with a decrease in lean tissue. No difference in the percentage of body fat was observed between RD and HFD-fed mice injected with either single or repeated LPS injections.

The body length of both RD and HFD-fed mice was similar and the growth in these mice was not impaired by single or repeated LPS injections. Results obtained from DEXA scans suggested that BMC and BMD in RD-fed mice were decreased with repeated LPS injections. On the other hand, no effect of LPS inflammatory challenge

was observed on bone remodeling in HFD-fed mice. Comparison between the two diet-fed groups indicated that BMC and BMD were decreased in saline-treated HFD-fed mice. Increased bone resorption has been observed in mice fed with a high-fat diet [587] and this observation supports the current findings. Repeated LPS injections reduced BMD and BMC in RD-fed mice but LPS treatment tended to show opposite effect in HFD-fed mice. It is unclear if some other metabolic factors could have had protective role in HFD-fed mice and this should be explored in future studies.

Glucose tolerance was not impaired in both RD and HFD-fed mice by single or repeated LPS inflammatory challenges. As it was described in previous studies, high-fat diet fed mice are considered as a robust model for glucose intolerance and type-2 diabetes [588]. Similarly, in the current study, comparison between the two diet-fed groups revealed that HFD-fed mice were glucose intolerant in groups injected with either saline or single LPS injection, whereas there was a tendency for five LPS-injected HFD-fed mice to have impaired glucose tolerance.

There was a trend for HFD-fed mice to have higher levels of plasma insulin than RD-fed mice and in particular it reached significance in the saline-treated group. In a similar manner, leptin level was elevated in HFD-fed mice and its plasma concentration was related with the percentage of fat tissue. LPS inflammatory challenge did not alter either of the insulin or leptin plasma levels. Based on the data collected from IPGTT, elevated levels of plasma insulin suggest that HFD-fed mice were insulin resistant. These observations are in agreement with previous studies that have demonstrated that a high-fat diet induces insulin resistance and this was associated with impaired expression of lipase in skeletal muscles [589].

Conclusions:

Aim 1: Long-term effect of systemic LPS challenge on metabolic outcomes, exerted through the IL-1 pathway.

- Deletion of caspase-1 and IL-1ra signaling contributed to increased food intake. Repeated LPS injections reduced the food intake in IL-1ra-/- mice, whereas CNS central control over food intake was not impaired in Casp-1-/- mice.

- Increased food intake did not contribute to weight gain nor excess fat mass accumulation in either Casp-1-/- or IL-1ra-/- mice. In particular fat mass accumulation was decreased in absence of the active role of IL-1ra. Casp-1-/- mice were protected against LPS-induced decreases in both body weight gain and fat depots accumulation, whereas fat accumulation in IL-1ra-/- was so low that probably it could not be further decreased by LPS intervention.
- Absence of caspase-1 signaling contributed to increased BMD and BMC, whereas LPS-induced decrease of BMD and BMC was independent of caspase-1 mediated IL-1β signaling pathway.
- Absence of IL-1ra signaling contributed to better insulin sensitivity probably due to increased metabolism, whereas absence of caspase-1 signaling did not improve the insulin sensitivity in Casp-1-/- mice. On the other hand, an excess of IL-1 signaling induced by LPS treatment reduced the insulin sensitivity in IL-1ra-/- mice, whereas Casp-1-/- mice were not affected.
- Absence of caspase-1 signaling contributed to the increased secretion of insulin, whereas insulin secretion was decreased in absence of IL-1ra. Over-stimulation of IL-1 signaling by LPS inflammatory challenge did not impair insulin in either Casp-1-/- or IL-1ra-/- mice.
- Lack of caspase-1 signaling contributed to reduced leptin secretion in Casp-1-/- mice, whereas very low leptin secretion in IL-1ra-/- mice was attributed to the very low amount of accumulated fat mass. LPS inflammatory challenge did not alter the leptin level.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on metabolic outcomes.

- High-fat diet consumption contributed to increased food intake. Repeated LPS inflammatory challenge increased the food intake in RD-fed mice, whereas high-fat diet did not exacerbate the LPS-induced inflammatory effect on food intake.
- Despite higher food intake, final body weights in HFD-fed mice were not increased. However, HFD mice tended to have increased fat mass accumulation. Repeated LPS inflammatory challenge tended to reduce weight gain and body fat mass accumulation in both RD and HFD-fed mice.

- Longitudinal growth was not altered by high-fat consumption nor was it exacerbated by LPS inflammatory challenge. On the other hand, high-fat diet consumption tended to reduce BMD and BMC, whereas systemic LPS challenge had tendency to display opposite effect in HFD-fed mice.
- High-fat diet consumption was associated with glucose intolerance that was not exacerbated by LPS inflammatory challenge as mice tended to lose weight.
- High-fat diet consumption resulted in increased plasma levels of insulin and leptin, which was not exacerbated by LPS inflammatory challenge.

5. Effect of peripheral inflammatory challenge and high-fat diet on the degeneration of dopamine neurons

5.1 Introduction

The process of neuroinflammation in the substantia nigra is characterized by the activation of microglia cells [283], increased levels of proinflammatory cytokines (e.g. IL-1β) [590] and chemokines such as MCP-1. In particular, IL-1 has been shown to be the key inflammatory factor involved in many neurodegenerative diseases [591]. Neuroinflammation has been considered to play a major role in the neurodegeneration of dopaminergic neurons since it was observed that degeneration of these neurons occurs in an idiopatic manner in 90% of Parkinson`s disease patients [592]. According to epidemiological and postmortem studies, a neuroinflammatory process was suggested to contribute to the progression of Parkinson`s disease [593]. Studies conducted in rodents suggest that chronic exposure to neurotoxins may predispose dopamine neurons to degeneration with advanced aging [594]. In a similar manner, epidemiological studies have suggested possible implications of pesticides in the pathogenesis of Parkinson`s disease [595].

Degeneration of dopaminergic neurons in rodents may be induced by several toxins (chapter 1.9) such as LPS, whose mechanism of action is established through the process of neuroinflammation. In regard to other inflammatory factors, IL-1β and TNF-α are the main proinflammatory cytokines considered to be involved in LPS-induced neurodegeneration of dopamine neurons [596]. As previously described in chapter 1.10.3, these cytokines (i.e. IL-1β and TNF-α) may affect dopamine neurons directly by inducing programmed cell death, or indirectly through activation of microglial cells (chapter 1.10.5.1). The LPS-induced neurotoxicity was first introduced in *in vitro* studies with rat mesencephalic cultures where it was shown that dopaminergic neurons were more susceptible to neurodegeneration than the other neuronal cells and this LPS-mediated toxicity occurred via activation of microglia [239, 596]. Moreover, a recent *in vivo* study conducted on mice

demonstrated that intracerebral infusion of LPS selectively induced degeneration of dopaminergic neurons without affecting cholinergic and serotonergic neurons [238].

The degree of dopaminergic neurodegeneration and duration of neuroinflammatory process varies according to the routes of injection and genotype, doses of LPS administration and age of the tested rodents. For example, it has been demonstrated that acute intranigral LPS injection (2μg) produces irreversible loss of dopamine neurons in SNpc within 21 days [597], but further decline has not been observed after a year from post-treatment [477]. In parallel with the previous statement, acute LPS intervention is accompanied with rapid activation of microglial cells but these cells revert to their ramified state in a very short period of time (30 days) [598]. On the other hand, chronic intranigral infusion of low doses of LPS (5ng) in rats produced delayed and progressive loss of dopaminergic neurons with significant activation of microglial cells [476]. In comparison with the inflammation induced directly in the CNS, systemic inflammation has been described as affecting the activity of resident immune cells in the brain that may be associated with a chronic neuroinflammatory state [599]. As described before in chapter 3.2, single systemic LPS injection (i.p.) induced the loss of dopamine neurons in the substantia nigra of male mice by 23% after 7 months and the loss progressed to 47% after 10 months [109]. Moreover, degeneration of dopamine neurons is exacerbated with higher doses of LPS, meaning that the degree of loss is also dose dependent [600]. Nigrostriatal injection of LPS produced greater loss of dopamine neurons in sixteen months old rats in comparison to younger ones (3 months old) [601], which suggest that older animals are more predisposed to neurodegeneration than younger animals. Collectively, these observations suggest that intranigral chronic neuroinflammation and systemic LPS administration may play a vital role in prolonged and significant loss of dopamine neurons [109, 599] that could further be exacerbated with higher dose of LPS [600] and in older experimental group of rodents [601].

As previously described in chapter 3.2, high-fat diet consumption and metabolic perturbations (chapter 4) that occur as a result of obesity may exacerbate the proinflammatory state induced by LPS and consequently alter the proper function of the nigrostriatal system. A recent study has shown that two months of high-fat diet feeding exacerbated the decrease of dopamine in the striatum in the MPTP-induced model of dopaminergic neurodegeneration [351]. Furthermore, it has been

demonstrated that mice fed a high-fat diet for 22 weeks had an increased level of proinflammatory proteins in the brain [602]. Even though there was no detectable difference in the number of CD68-positive microglial cells (measured as an index of microglia activation), it has been additionally observed that isolated microglial cells from high-fat diet-fed mice secreted more TNF-α in comparison to chow diet-fed mice [602]. These papers suggest that the inflammatory profile in the brain may be up-regulated with high-fat diet-induced obesity and possibly exacerbated the loss of dopamine neurons [351], which does not have to be necessarily correlated with the number of activated microglia [602].

Down-regulation of the neuroinflammatory process ameliorates the loss of dopamine neurons. For instance, down-regulation of inflammation by ciclooxigenase inhibitor-2 has been shown to attenuate the dopamine cell loss in rodents [603]. In a similar manner, pharmacological administration of human recombinant IL-1 receptor antagonist (anakinra) attenuated the loss of dopamine neurons and reduced the level of IL-1 with the nigrostriatal injection of Toll-like receptor-3 agonist polyinosinic:polycytidylic acid in rats [158], suggesting that the neuroinflammatory process may play a major role in the progression of dopaminergic neurodegeneration.

5.2 Objectives

The aim of this chapter is to assess the effect of high-fat diet and LPS inflammatory challenge on activation of microglial cells and their concomitant role in the degeneration of dopamine neurons, measured by TH staining. Additionally, plasma level of MCP-1 have been measured in order to assess the proinflammatory profile in the peripheral organs and to ascertain if this inflammatory profile is related with the inflammatory status of the brain.

5.3 Brief methodology

Frozen mouse brains were cut on cryostat and processed for immunohistochemical analysis. Eight evenly spaced frozen sections that encompassed the entire region of substantia nigra were cut and stained with rabbit anti-tyrosine hydroxylase antibody, a positive marker of dopamine neurons and rat anti-mouse CD68 monoclonal antibody, a marker of activated microglia/macrophages. The unilateral side of the substantia nigra was used for quantification of TH-positive neurons and CD68-

positive microglial cells. Slides were quantified under the microscope and a total cell count of eight slides per animal was recorded as for the final result. In order to assess the plasma level of MCP-1 chemokine, an ELISA test was performed from the blood plasma.

5.4 Results

In order to assess the effect of LPS inflammatory challenge on neuronal degeneration of dopamine neurons, the total number of TH-positive neurons was quantified from eight evenly spaced frozen sections of the brain that encompassed the entire region of the substantia nigra.

In figure 5.1 (A, B and C), the total cell count of dopamine neurons of WT, Casp-1-/- and IL-1ra-/- mice is presented in order to assess the effect of single or repeated LPS treatment on neuronal degeneration. As it is presented in figure 5.1 A, single or repeated LPS injections did not significantly decrease the number of dopamine neurons in WT mice. Similarly, repeated LPS treatment did not exacerbate the loss of dopamine cells in the Casp-1-/- and IL-1ra-/- group of mice in comparison to their corresponding saline-treated groups (figure 5.1 B and C). For that reason the effect of single LPS injection on TH-positive cells was not further evaluated in Casp-1-/- and IL-1ra-/- groups.

In order to assess the role of the IL-1 pathway on dopaminergic neurodegeneration, the total count of TH-positive cells was compared among three different genotypes within the same treatment (figure 5.1 D). As presented inside the saline-treated group, the lack of IL-1ra led to a reduction of up to 24% of dopamine neurons in IL-1ra-/- mice in comparison to Casp-1-/- ($p<0.001$) and WT mice ($p<0.05$). Similarly, the same trend was observed among the three genotypes that received five LPS injections, suggesting that the loss of dopamine neurons in IL-1ra-/- mice was not additionally affected by LPS treatment (figure 5.1 D). A positive TH-staining of dopamine neurons in SNpc of WT, Casp-1-/- and IL-1ra-/- mice that were injected with saline, or five monthly LPS injections is presented in figure 5.2.

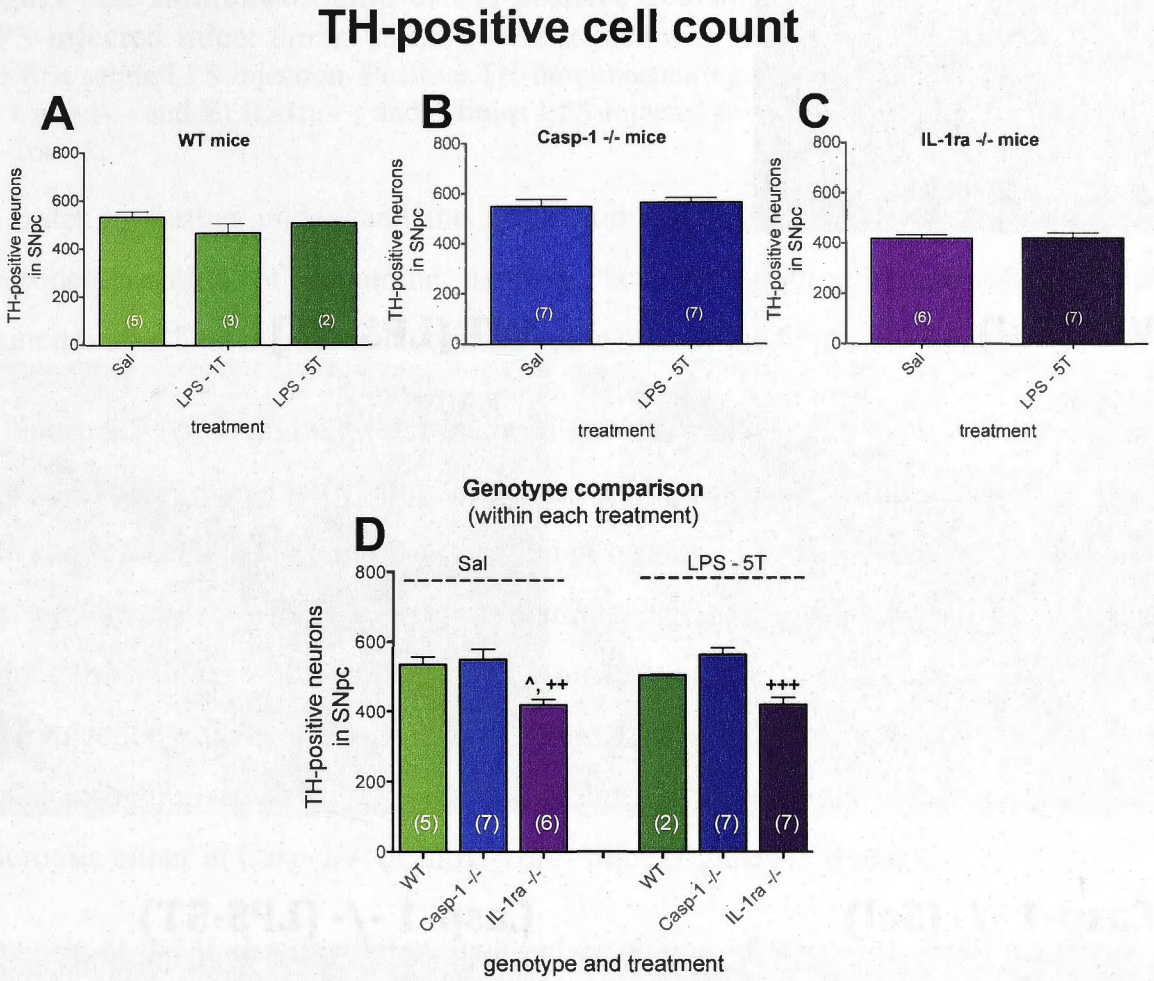
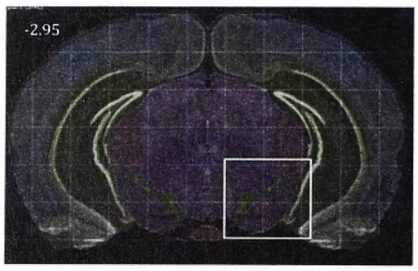
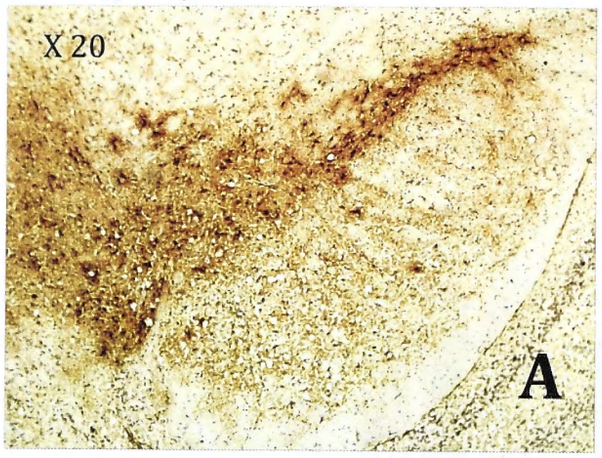


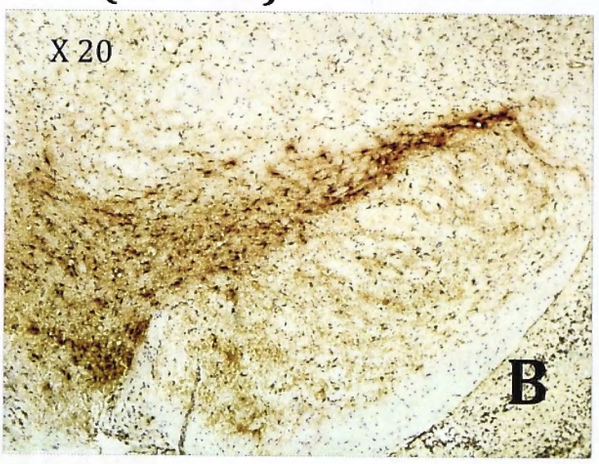
Figure 5.1: Effect of LPS inflammatory challenge on degeneration of dopamine neurons: Fifteen months after the first saline/LPS injections mice brains were harvested and 32 consecutive slides (rostral to caudal: -2.65 to -3.61 mm posterior from bregma) were collected. The total number of TH-positive cells from eight evenly spaced frozen sections were evaluated from the unilateral side of SNpc and presented individually by different genotypes A) WT, B) Casp-1-/- and C) IL-1ra-/- mice, in order to assess the effect of LPS treatment. Data were analyzed by one-way ANOVA (Tukey`s post-hoc test) and unpaired T-test. D) The total number of TH-positive neurons was compared among three different genotypes within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent the standard errors. ^$p<0.05$ vs. WT; $^{++}p<0.001$, $^{+++}p<0.0001$ vs. Casp-1-/-.



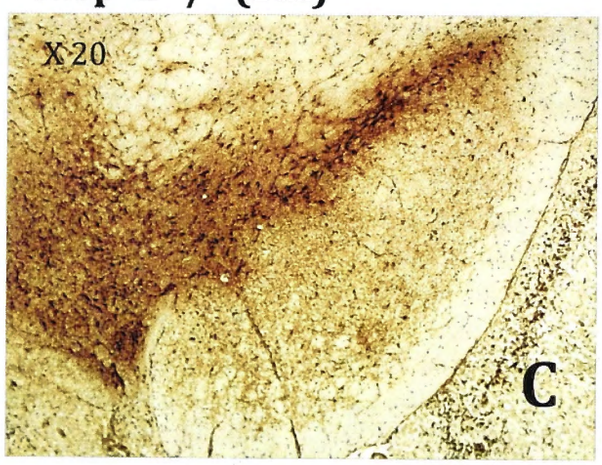
WT (Sal)



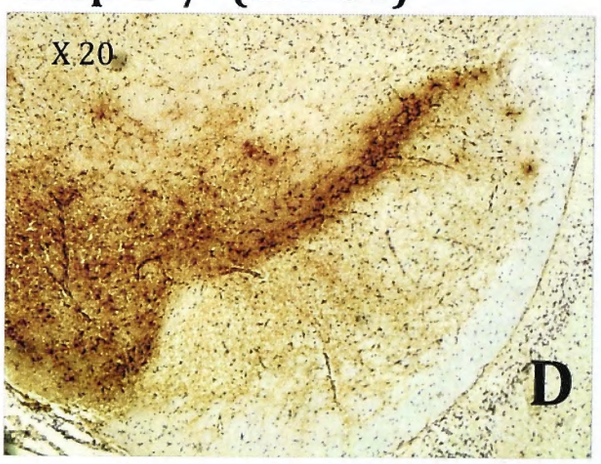
WT (LPS-5T)



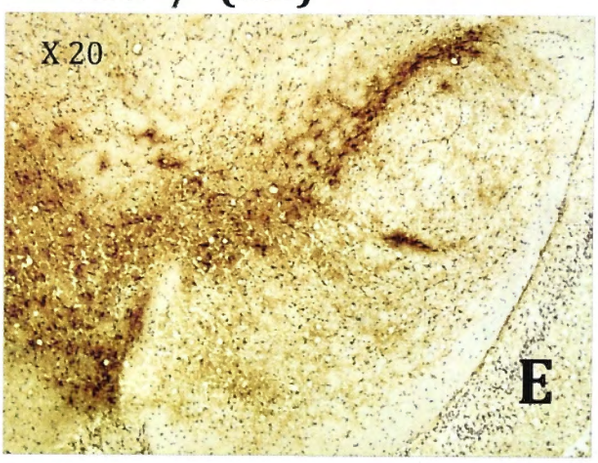
Casp-1 -/- (Sal)



Casp-1 -/- (LPS-5T)



IL-1ra -/- (Sal)



IL-1ra -/- (LPS-5T)

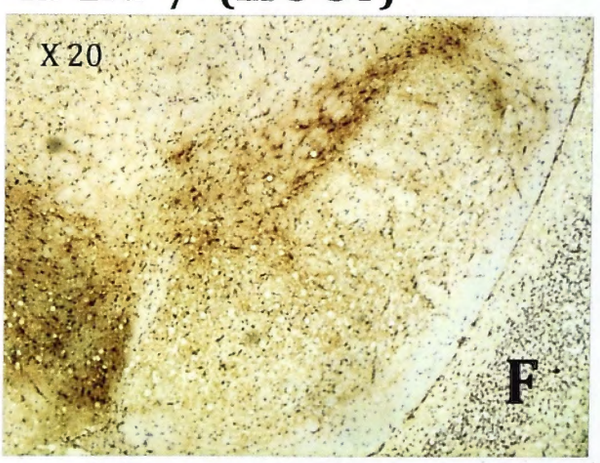


Figure 5.2: Immunostaining of TH-positive neurons in saline and five monthly LPS-injected mice: Frozen sections were stained with anti-TH antibody 15 months after the first saline/LPS injection. Positive TH-immunostaining of saline-injected mice: A) WT, C) Casp-1-/- and E) IL-1ra-/-; and 5-times LPS-injected mice: B) WT, D) Casp-1-/- and F) IL-1ra-/-.

In order to further understand the implication of activated microglial cells in the neurodegeneration of dopamine neurons, activated microglia/macrophages were stained with CD68 marker and counted in the area of the SNpc and SNpr.

In figure 5.3 (A, B and C), total count of CD68-positive cells of WT, Casp-1-/- and IL-1ra-/- mice treated with saline or repeated LPS injections is presented. The effect of a single LPS injection on the activation of microglia cells was not assessed since it was previously shown (figure 5.1) that single exposure to LPS did not significantly reduce the number of dopaminergic neurons. As presented in figure 5.3 A, repeated LPS injections did not significantly increase the number of activated microglia in WT mice. Similarly, five LPS injections did not increase the number of activated microglia either in Casp-1-/- or in IL-1ra-/- mice (figure 5.3 B and C).

The role of the IL-1 signaling pathway in activation of microglia cells was assessed in the three different genotypes and compared within the same treated group (figure 5.3 D). In the saline-treated group, Casp-1-/- mice had a significantly lower number of activated microglia (approximately 50%) in comparison to WT and IL-1ra-/- mice, suggesting that these mice show an overall lower inflammatory cascade within the brain. No significant difference between IL-1ra-/- and WT mice was observed. Similarly, the same trend in the activation of microglia cells was shown in groups treated with five LPS injections. These data suggest that IL-1 signaling was involved in the activation of microglial resident immune cells. A positive CD68-staining of activated microglia cells in the substantia nigra of WT, Casp-1-/- and IL-1ra-/- mice that were injected with saline, or five monthly LPS injections is presented in figure 5.4.

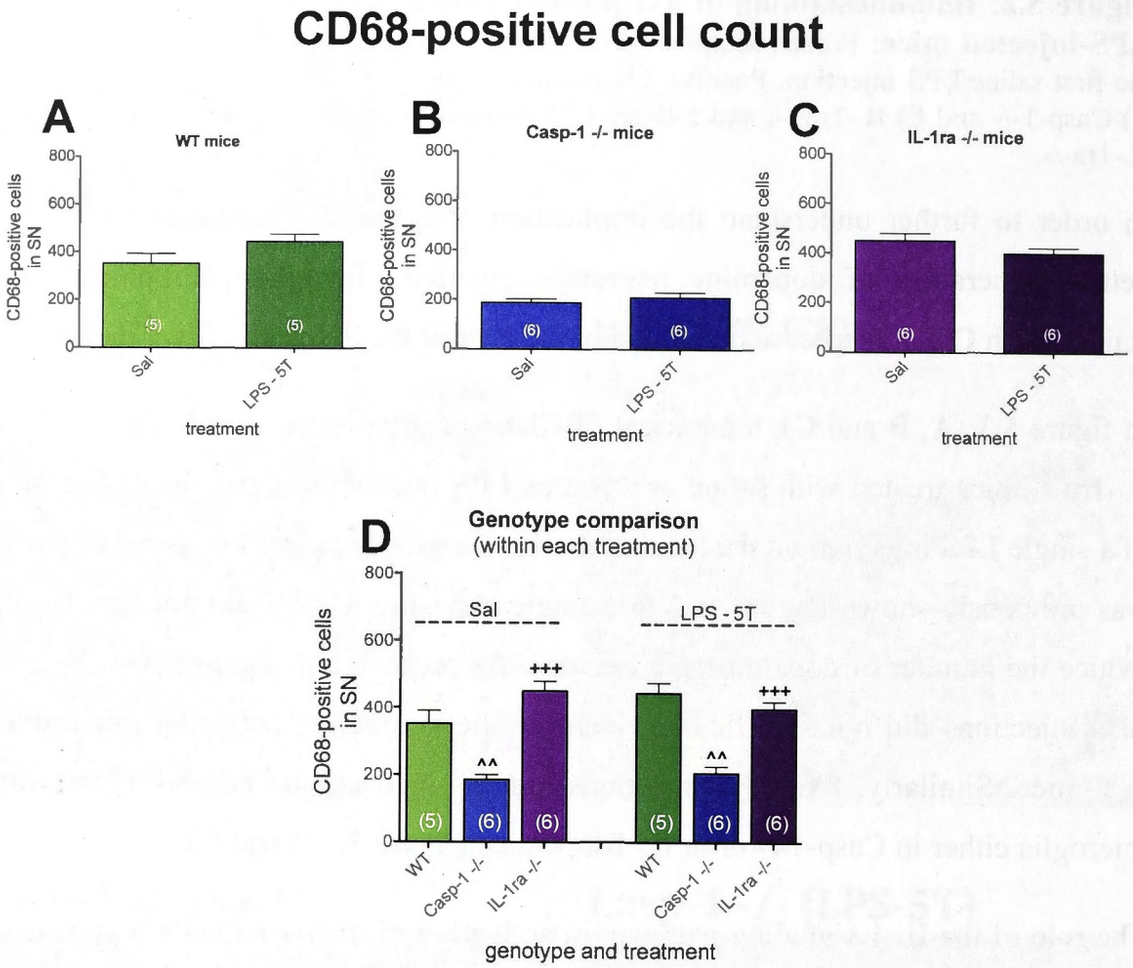
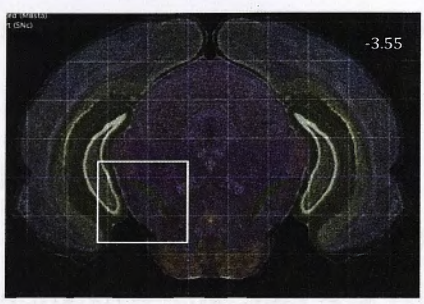
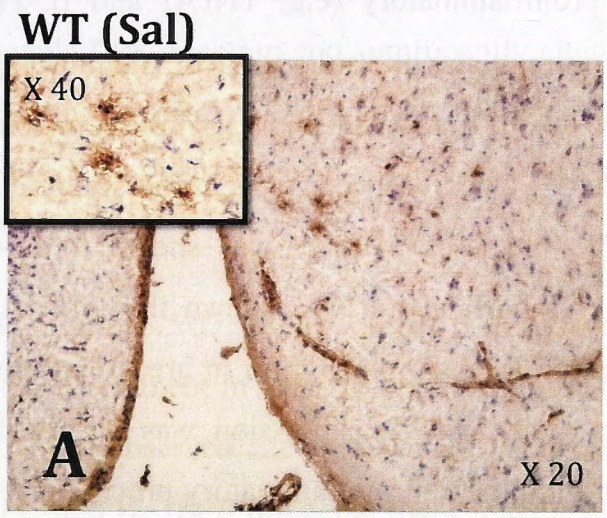
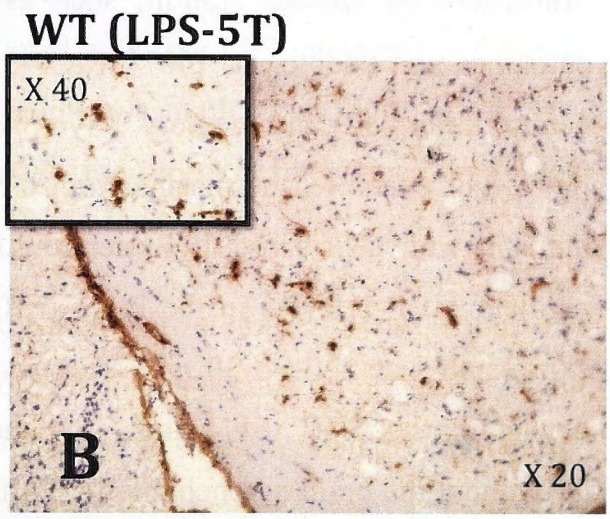
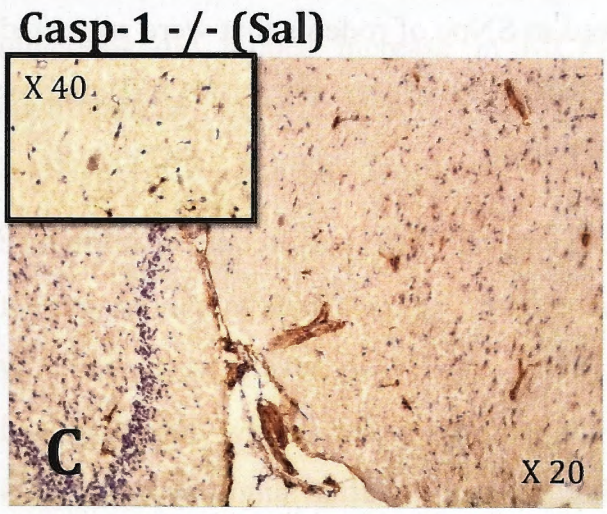


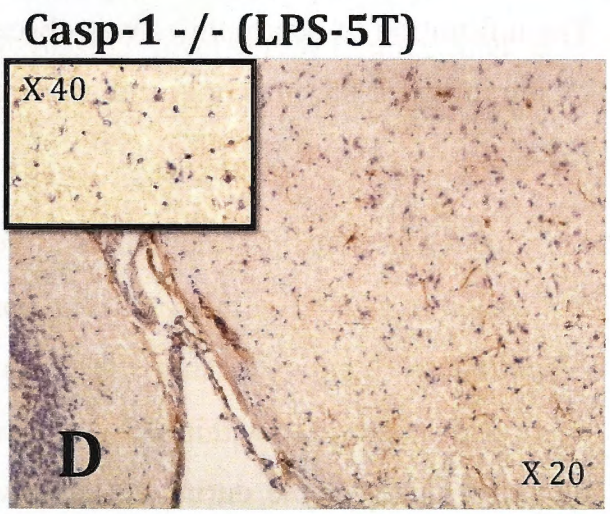
Figure 5.3: Effect of LPS inflammatory challenge on activation of microglia cells: Eight evenly spaced frozen sections that encompassed the entire substantia nigra were stained with CD68, a marker for activated microglia. Total count of CD68-positive cells were counted in the region of substantia nigra (SN) in groups of A) WT, B) Casp-1-/- and C) IL-1ra-/- mice and analyzed by unpaired T-test. D) Genotype comparison of the total number of CD68-positive cells was analyzed within each treatment by one-way ANOVA followed by Tukey`s post-hoc test. Error bars represent standard errors. ^^$p<0.001$ vs. WT; $^{+++}p<0.0001$ vs. Casp-1-/-.

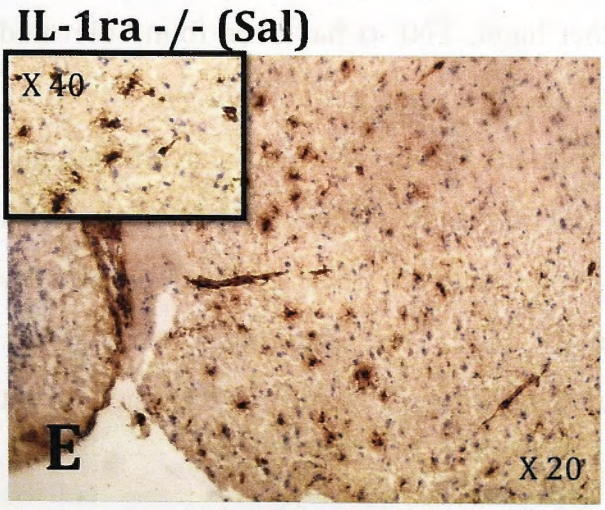

-3.55


WT (Sal)
X 40
A
X 20


WT (LPS-5T)
X 40
B
X 20


Casp-1 -/- (Sal)
X 40
C
X 20


Casp-1 -/- (LPS-5T)
X 40
D
X 20


IL-1ra -/- (Sal)
X 40
E
X 20

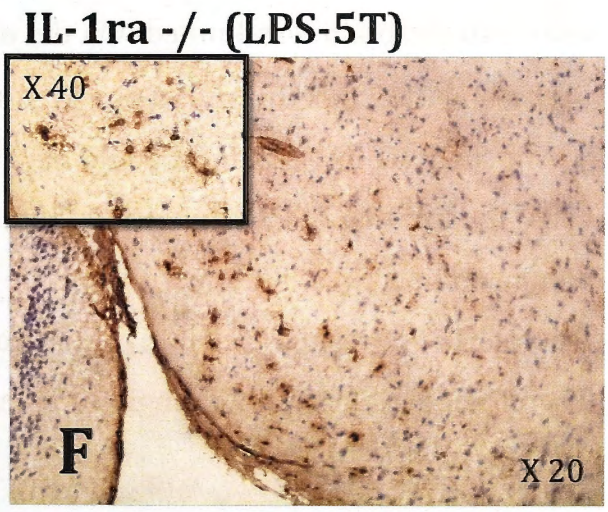

IL-1ra -/- (LPS-5T)
X 40
F
X 20

Figure 5.4: Immunostaining of CD68-positive microglia cells in saline and five monthly LPS-injected mice: Frozen sections were stained with anti-CD68 antibody 15 months after the saline/LPS injection. Positive CD68-immunostaining in saline-injected mice: A) WT, C) Casp-1-/- and E) IL-1ra-/-; and 5-times LPS-injected mice: B) WT, D) Casp-1-/- and F) IL-1ra-/-.

MCP-1 is a chemokine produced by a variety cells types such as endothelial cells, epithelial cells, fibroblast, astrocytes, smooth muscle and microglia [604-606]. Stimulated by various stimuli, such as proinflammatory (e.g. TNF-α and IL-1) cytokines and endotoxins [607], MCP-1 up-regulates the migration of monocytes, natural killer cells and memory T lymphocytes to the site of injury or inflammation [608]. For example, the plasma level of MCP-1 has been elevated in the serum of humans with sepsis [609]. Systemic LPS injections in mice was shown to up-regulate MCP-1 levels in the brain [610]. In particular it was shown that MCP-1 expression was noticed 30 min after systemic LPS administration in areas that are devoid of the BBB [611] and 6-8 hours later, MCP-1 expression was observed throughout the brain parenchyma [612]. In turn, the neuroinflammatory process may contribute to the infiltration of immune cells from the periphery into the SNpc [613]. The infiltration of neutrophiles was observed in SNpc of rodents that were subjected to intranigral LPS injection and were detected by expression of MCP-1 marker [613].

In mice with hepatic inflammation, peripheral TNF-α was required to induce expression of MCP-1 in microglia and concomitantly to contribute to the recruitment of peripheral monocytes into the brain [614]. As TNF-α levels were markedly decreased in plasma (within 3 hours) to the baseline point (below detection) and remained undetectable during the following ten months after single systemic LPS injection (5 mg/kg) [109, 403], it may be suggested that recruitment of local immune cells into the brain was limited. On the other hand, TNF-α has been found elevated in the brain ten months after a single systemic LPS injection (5 mg/kg) [109, 403], which suggests that this level could have originated from activated resident immune cells in the brain. In connection with the previous statement, peripheral LPS administration was reported to induce production of MCP-1 in the brain by macrophage/microglia cells [611], astrocytes [612] and endothelial cells [611]. Therefore, it is uncertain if single or repeated systemic LPS injections in the current

study could have up-regulated the MCP-1 for a longer period in the periphery and contributed to the infiltration of peripheral immune cells into the brain.

In this study, the level of MCP-1 was measured in the blood plasma in order to determine if MCP-1 in the periphery could affect changes in the brain. As presented in figure 5.5 A, MCP-1 levels increased, but not significantly, after repeated LPS injections in WT mice. In contrast, the concentration of MCP-1 was decreased with a single LPS injection and significantly attenuated with five LPS injections in Casp-1-/- mice (figure 5.5 B). Similarly to Casp-1-/- mice, plasma level of MCP-1 in IL-1ra-/- group was significantly decreased with single and repeated LPS injections (figure 5.5 C).

In figure 5.5 D, MCP-1 level is compared among different genotypes within the same treatment in order to understand the role of IL-1 in regulation of MCP-1. As it may be observed, the levels of MCP-1 were elevated in saline-treated group of Casp-1-/- and IL-1ra-/- mice, but there was no significant difference between genotypes. A similar trend was shown with a single LPS injection. However, repeated LPS injections did induce increase in MCP-1 plasma level in WT mice that was significant in comparison to Casp-1-/- mice. These observations suggest that the level of MCP-1 in the periphery is not tightly regulated by the IL-1 signaling pathway.

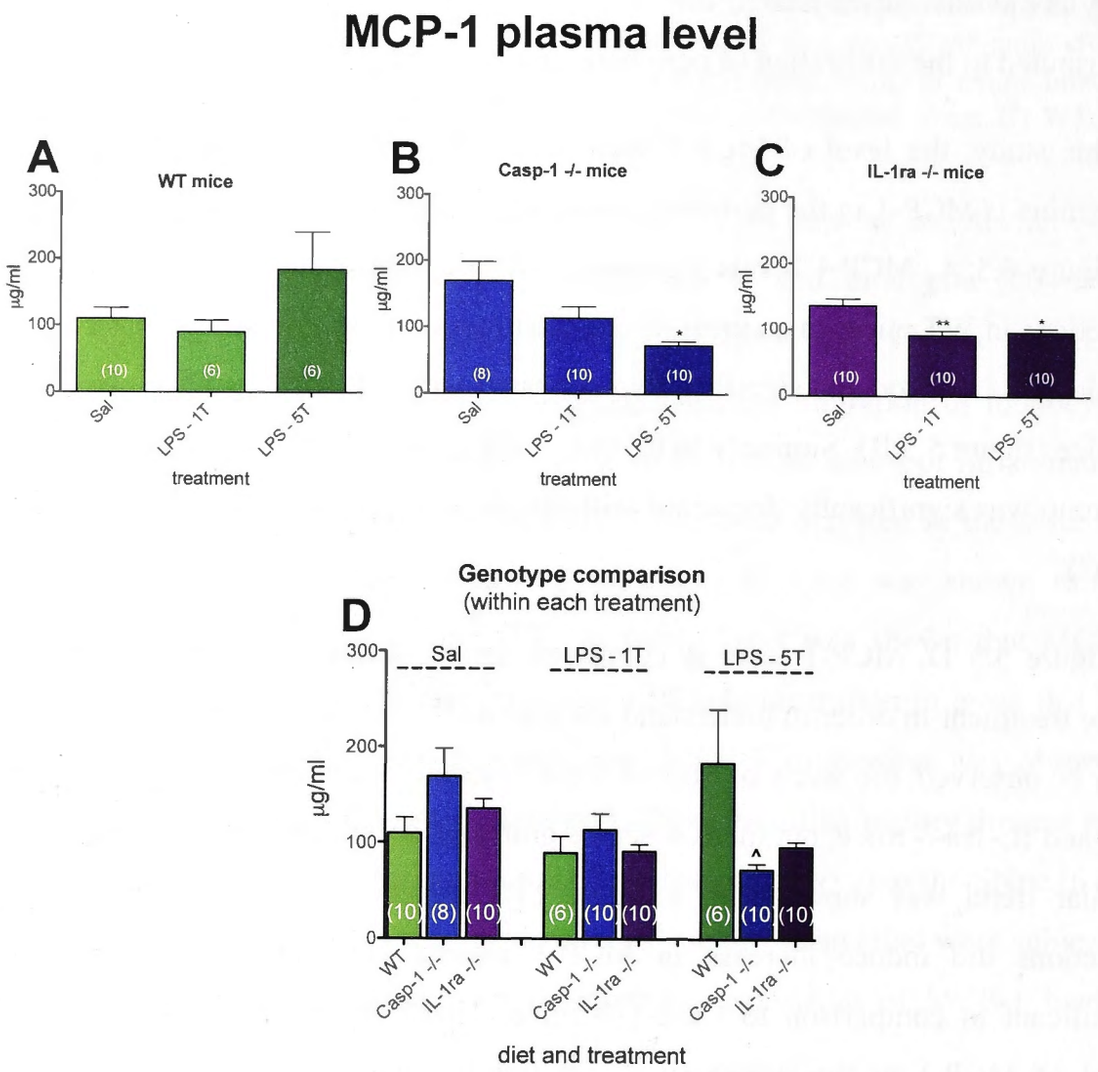


Figure 5.5: Plasma level of MCP-1 chemokine: The MCP-1 level was measured from blood plasma and concentration was expressed in µg/ml. The effect of LPS treatment on MCP-1 level in A) WT, B) Casp-1-/- and C) IL-1ra-/- mice was analyzed by one-way ANOVA (Tukey`s multiple comparison test). D) Level of MCP-1 was compared among different genotypes within same treated group by one-way ANOVA followed by Tukey`s post-hoc test. *$p<0.05$, **$p<0.01$ vs. Sal, ^$p<0.05$ vs. WT.

In order to assess the effect of LPS treatment and a high-fat diet on the degeneration of dopamine neurons, groups of mice fed either a regular or high-fat diet were analyzed separately in figure 5.6 (A and B). As it may be observed in WT mice fed a regular diet, single and repeated LPS injections tended to reduce the total number of dopamine neurons, but this was not significant (figure 5.6 A). On the other hand, repeated LPS injections did induce significant loss of dopamine neurons by 11% in high-fat diet-fed mice ($p<0.05$) (figure 5.6 B).

Data of both groups were collapsed from figures 5.6 A and B in order to evaluate the additional effect of high-fat diet consumption on the loss of dopamine neurons. As may be observed in figure 5.6 C, there was not a significant reduction in dopamine

neurons in saline or LPS-treated groups, suggesting that the loss of TH-positive neurons was not induced or exacerbated by long-term high-fat diet consumption. Similarly, quantification of TH-positive cells in SNpc of HFD-NI mice, yielded no differences (data not shown). A positive TH-staining of dopamine neurons in SNpc of RD and HFD-fed mice that were injected with saline, or five monthly LPS injections is presented in figure 5.7.

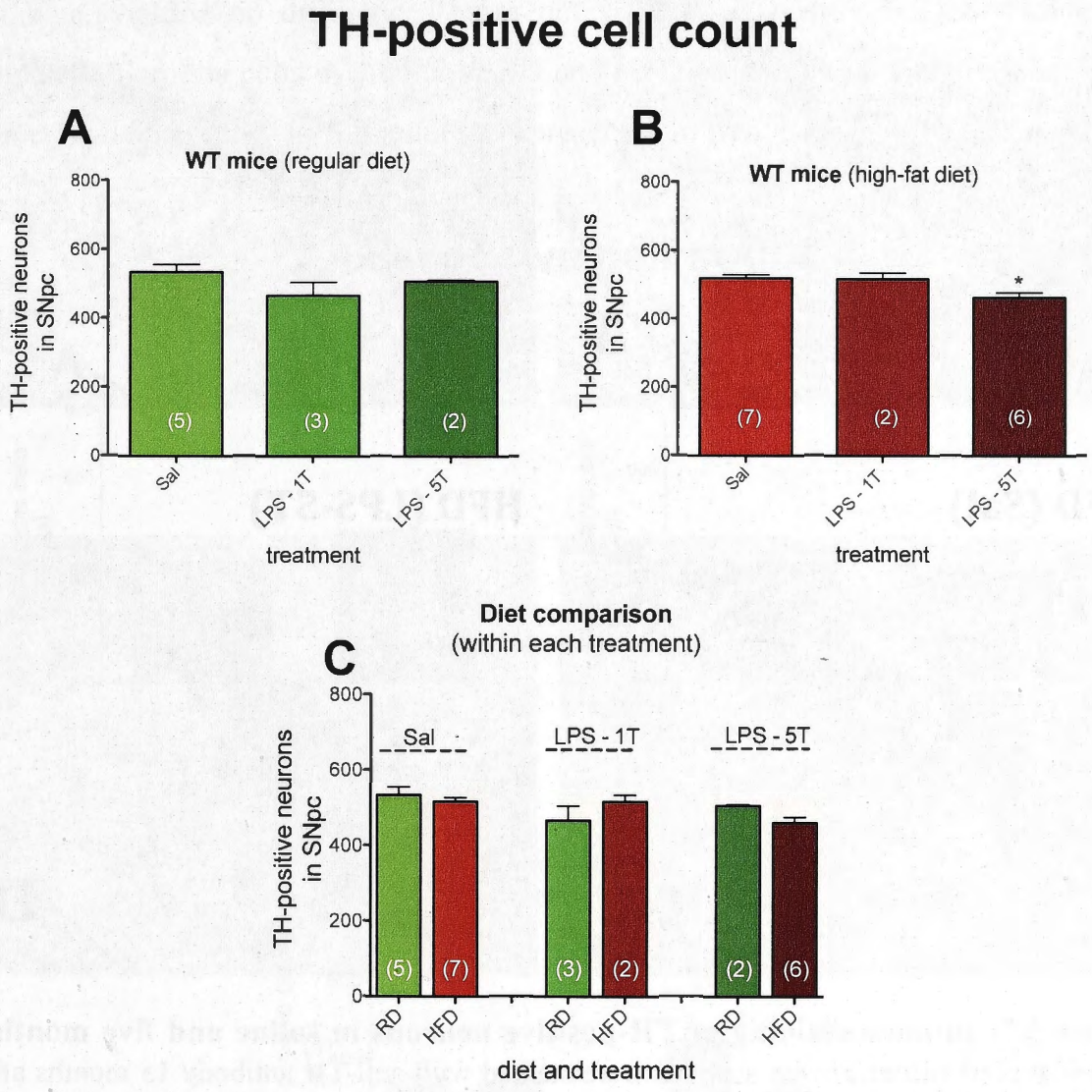
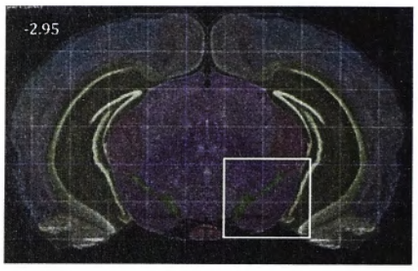


Figure 5.6: Effects of high-fat diet and LPS inflammatory challenge on the loss of dopamine neurons: Total number of TH-positive cells from eight evenly spaced frozen sections were evaluated from the unilateral side of SNpc and presented individually by diets A) RD and B) HFD-fed mice, in order to assess the effect of LPS treatment. Data were analyzed by one-way ANOVA (Tukey's post-hoc test). D) Total number of TH-positive neurons was compared between two different diets within each treatment by unpaired T-test. Error bars represent the standard errors. *$p<0.05$ vs. Sal.



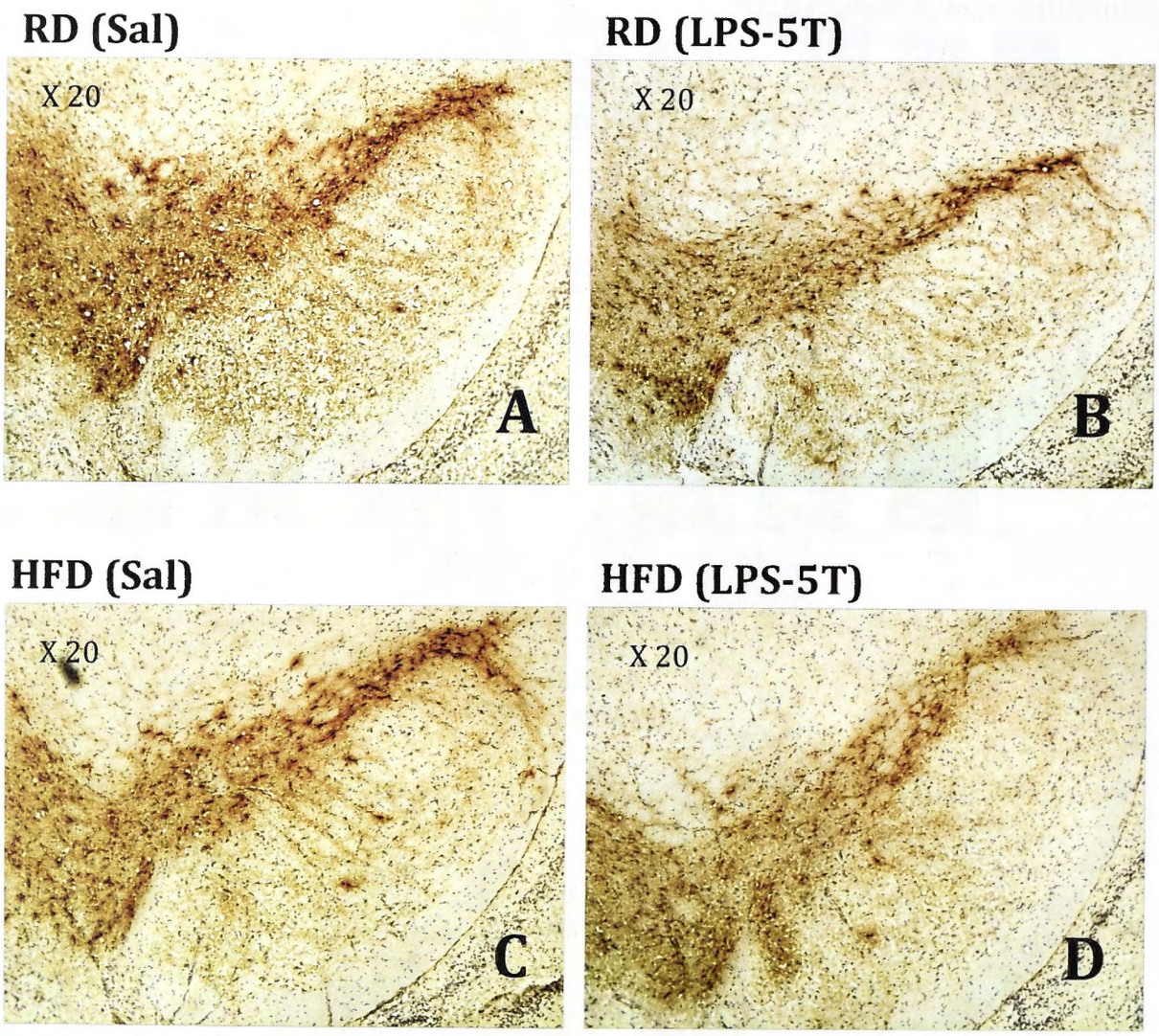


Figure 5.7: Immunostaining of TH-positive neurons in saline and five monthly LPS-injected mice: Frozen sections were stained with anti-TH antibody 15 months after first saline/LPS injection. Positive TH-immunostaining of saline-injected mice: A) RD and C) HFD; and 5-times LPS-injected mice: B) RD and D) HFD.

In order to assess the long-term effect of high-fat diet consumption and LPS treatment on microglia activation, frozen brain sections were stained with CD68 microglia marker and quantified. In figures 5.8 A and B the effect of repeated LPS injections was assessed separately in RD and HFD groups. Repeated LPS inflammatory challenge increased, but not significantly, the activation of microglial cells in RD-fed mice whereas this effect was not observed in HFD-fed mice.

In figure 5.8 D, groups of mice fed with two different diets were compared within same treatment in order to evaluate the effect of a high-fat diet in the activation of microglia cells. As may be observed, the total number of activated microglia cells in the SN region was not changed in saline-treated groups, but it was significantly increased in RD-fed mice injected with five LPS injections. These observations suggest that a high-fat diet did not induce or exacerbate the activation of microglia cells. Similarly, quantification of CD68-positive cells in the substantia nigra of HFD-NI mice, yielded no differences (data not shown). A positive CD68-staining of activated microglia cells in the SN of RD and HFD-fed mice that were injected with saline, or five monthly LPS injections is presented in figure 5.9.

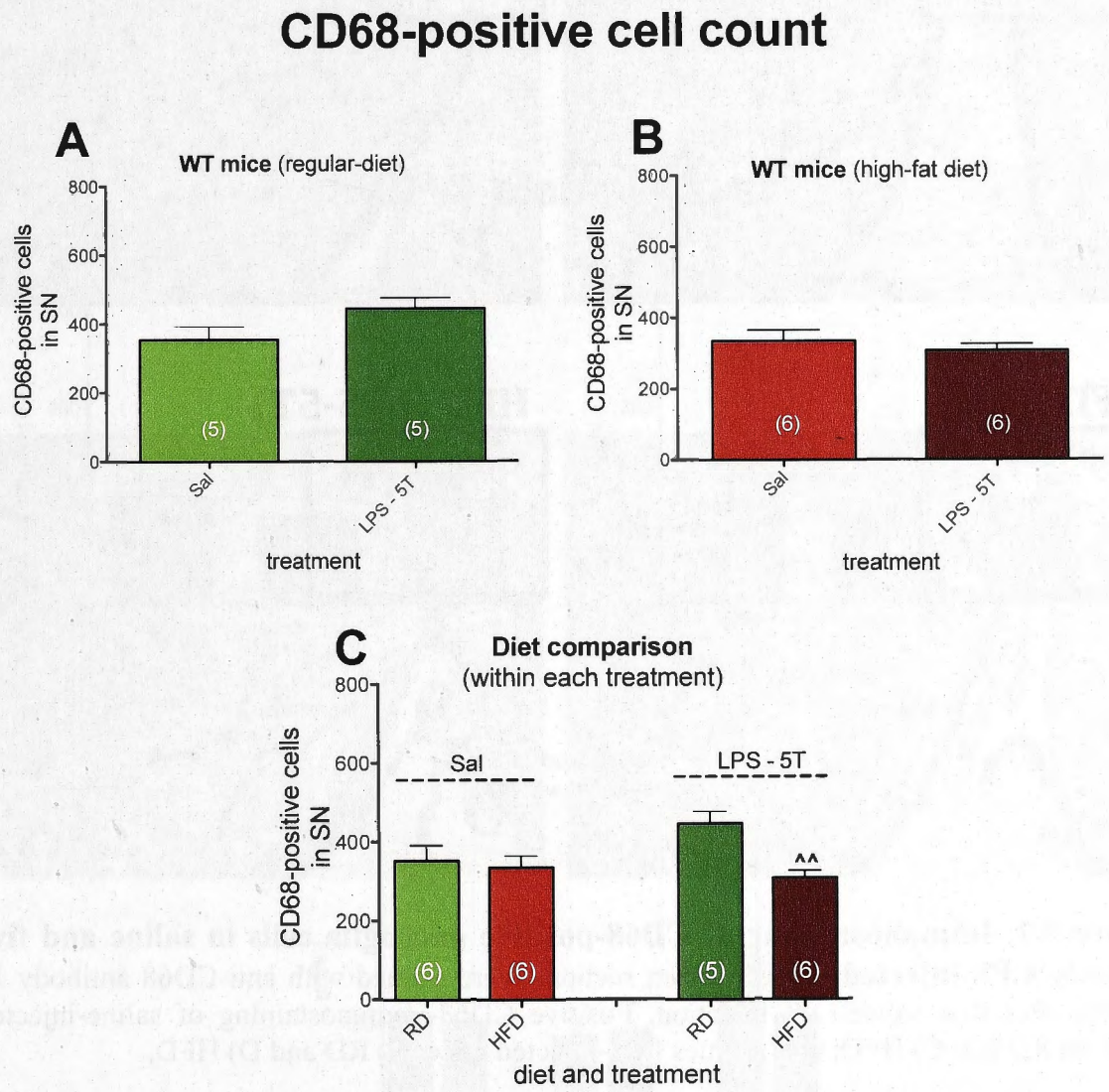
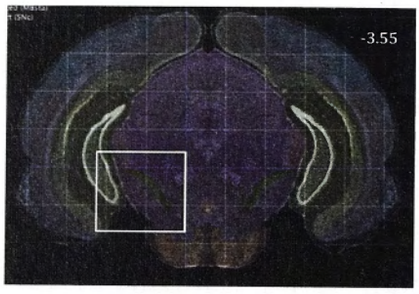


Figure 5.8: Effects of high-fat diet and LPS inflammatory challenge on the activation of microglia cells: Eight evenly spaced frozen sections that encompassed the entire region of the SN were stained with CD68, marker for activated microglia. The total number of CD68-positive cells was counted in the region of the SN in groups fed A) RD and B) HFD and analyzed by unpaired T-test. D) Comparison of total number of CD68-positive

cells between groups fed with different diets and within each treatment. Data were analyzed by unpaired T-test. Error bars represent standard errors. ^^p<0.001 vs. RD.



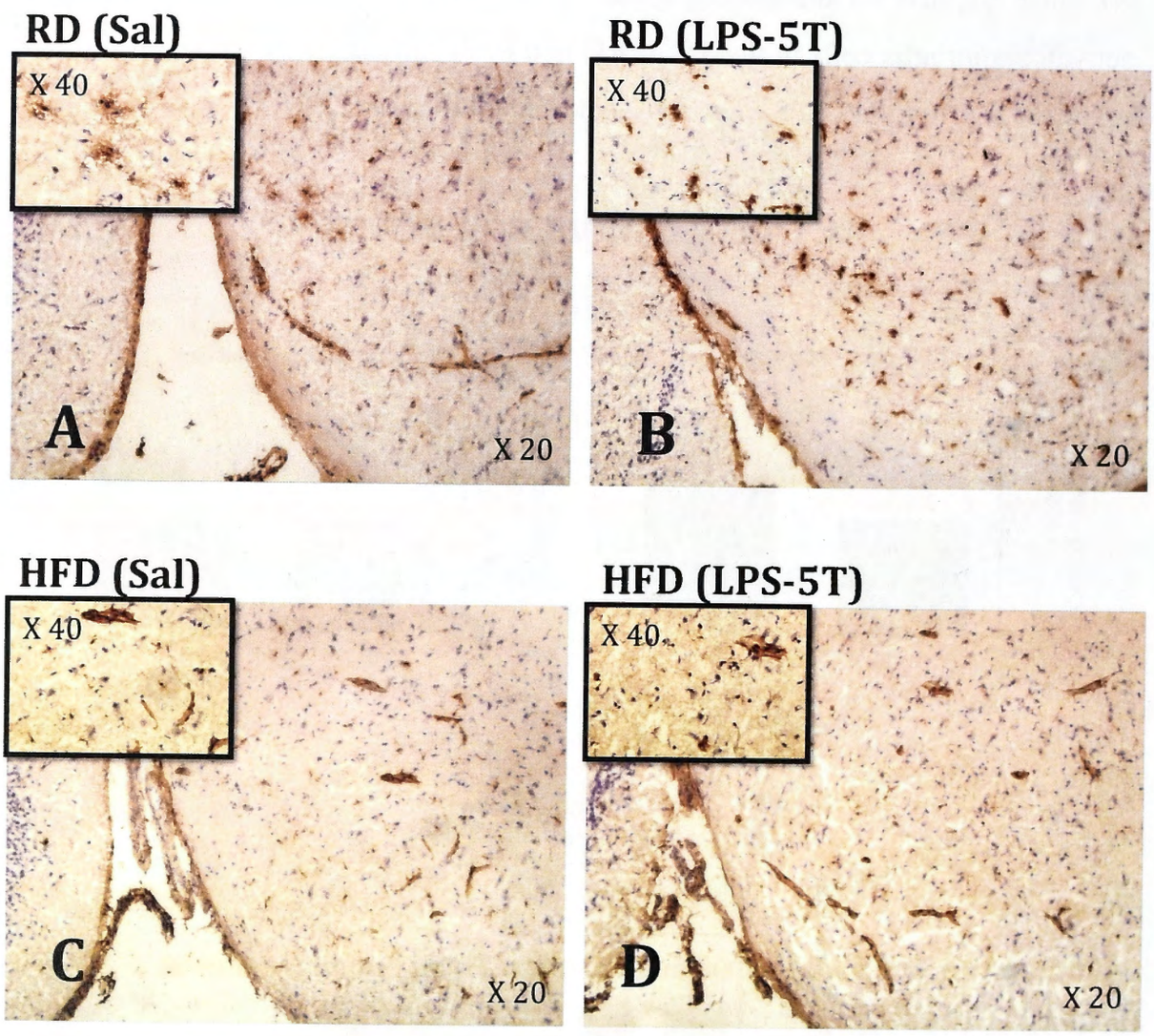


Figure 5.9: Immunostaining of CD68-positive microglia cells in saline and five monthly LPS-injected mice: Frozen sections were stained with anti-CD68 antibody 15 months after first saline/LPS injection. Positive CD68-immunostaining of saline-injected mice: A) RD and C) HFD; and 5-times LPS-injected mice: B) RD and D) HFD.

The effect of LPS inflammatory challenge and high-fat diet on plasma level of MCP-1 was assessed in order to better understand a possible connection of peripheral inflammation with an inflammatory outcome in the CNS.

In figures 5.10 A and B, the effect of single and repeated LPS injections on MCP-1 levels was evaluated in mice fed with either RD or HFD. Repeated LPS inflammatory challenge increased, but not significantly, the level of MCP-1 in RD-fed mice, while a single LPS injection did not produce any effect (figure 5.10 A). Long-term exposure to high-fat diet did not increase the level of MCP-1 in concert with single or repeated LPS inflammatory challenges (figure 5.10 B).

Comparison between groups fed with different diets and within the same treatment (figure 5.10 C) revealed that levels of MCP-1 were similar in both HFD and RD-fed mice injected with either saline or a single LPS injection. The only difference observed was in five times LPS injected mice where RD-fed mice had elevated levels of MCP-1 in comparison to HFD-fed mice, but this was not statistically different.

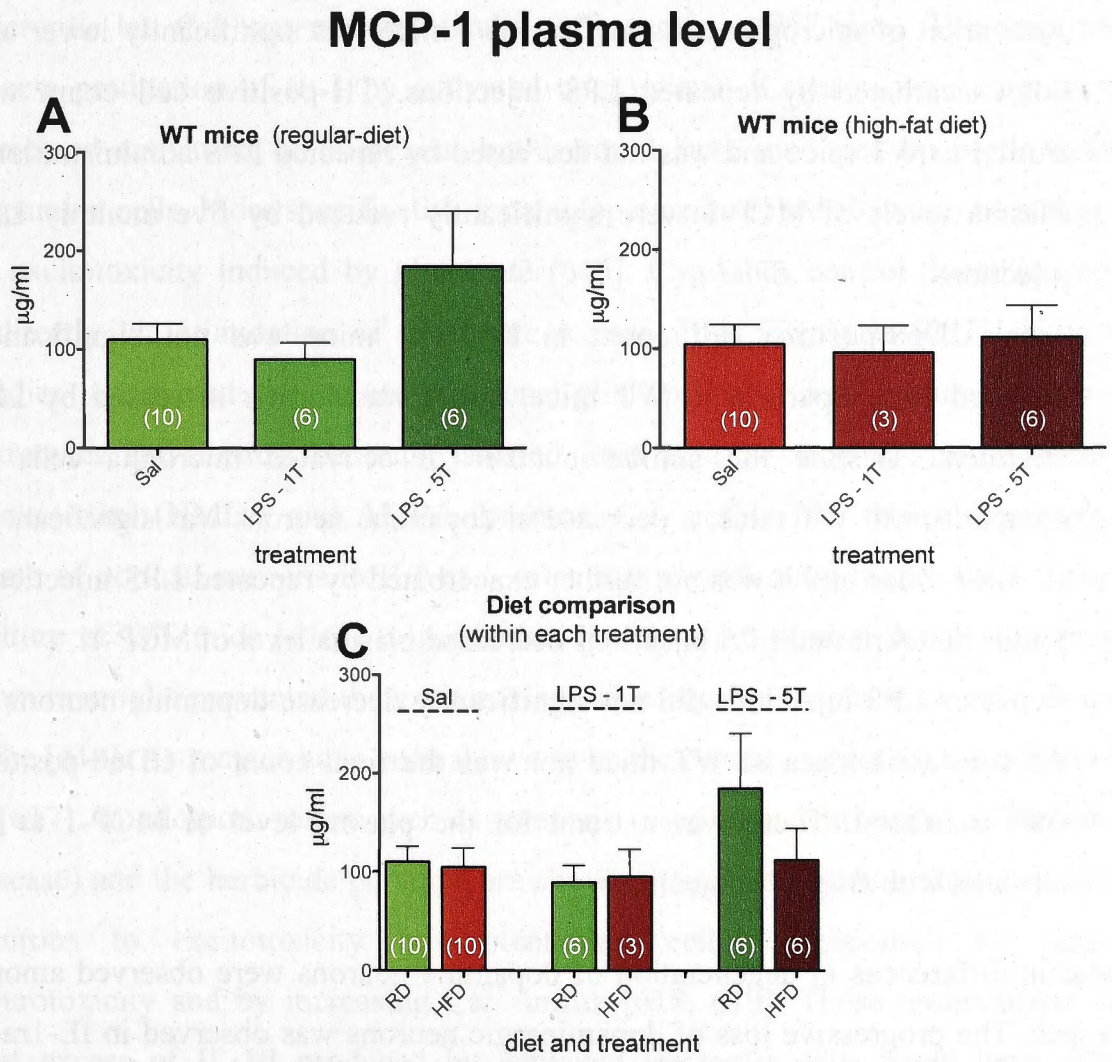


Figure 5.10: MCP-1 level in plasma: The MCP-1 level was assessed from blood plasma and concentration was expressed in µg/ml. The effect of LPS treatment on MCP-1 levels in the group of mice fed either A) RD or, B) HFD was analyzed by one-way ANOVA

(Tukey`s multiple comparison test). D) Level of MCP-1 was compared between groups fed with different diets and within the same treatment by unpaired T-test. Error bars represent standard errors.

5.5 Discussion

For this study to determine the role of the IL-1 signaling pathway and possible exacerbation of systemic LPS inflammatory challenge by high-fat diet on prolonged and progressive degeneration of dopamine neurons, the experimental model previously described by Yuxin Liu, et al. was used [376].

AIM 1: Long-term effect of systemic LPS challenge on inflammatory outcomes, exerted through the IL-1 pathway.

Major differences in activation of microglia cells and final cell count of dopamine neurons were observed among the three genotypes (WT, Casp-1-/- and IL-1ra-/-). On the other hand, the effect of LPS treatment was modest or undetectable.

- Activation of microglia cells in Casp-1-/- mice was significantly lower and not exacerbated by repeated LPS injections. TH-positive cell count was similar to WT mice and was not decreased by repeated LPS administrations. Plasma levels of MCP-1 were significantly reduced by five monthly LPS injections.
- Total CD68-positive cell count in IL-1ra-/- mice was not significantly elevated in comparison to WT mice, nor it was further increased by LPS treatment. Despite the similar number of activated microglia cells in comparison to WT mice, a decrease in dopamine neurons was significant in IL-1ra-/- mice and it was not further exacerbated by repeated LPS injections. Single and repeated LPS injections decreased plasma level of MCP-1.
- Repeated LPS injections did not significantly decrease dopamine neurons in the substantia nigra of WT mice nor was the final count of CD68-positive cells increased. There was a trend for the plasma level of MCP-1 to be elevated with five LPS injections.

Significant differences in degeneration of dopamine neurons were observed among genotypes. The progressive loss of dopaminergic neurons was observed in IL-1ra-/- mice, whereas dopamine neurons remained relatively intact in Casp-1-/- mice, indicating the relevance of the IL-1 signaling pathway in neurodegeneration. In

comparison to the previously described model [376], single and repeated LPS injections reduced the number of TH-positive neurons in WT mice but this loss was not significantly different to their corresponding saline-treated group. The modest loss of TH-positive neurons with five monthly LPS injections in WT mice might have been accompanied by a slightly increased number of activated microglial cells. On the other hand, it is unclear if the increased level of MCP-1, (increased but not statistically different compared with saline-injected WT mice), could have contributed to the elevated number of microglial cells.

Degeneration of dopamine neurons in IL-1ra-/- mice was not further exacerbated with five monthly administrations of LPS. This observation might suggest that the 24% loss of dopamine neurons in both saline and LPS-treated groups could not be further exacerbated with additional systemic inflammation challenge. The loss of neurons might have been accompanied with elevated cell numbers of activated microglia but this was not statistically different from WT mice. This implies that excess production of IL-1 by activated microglia in IL-1ra-/- mice may not have increased activation of microglia, but it could have mediated apoptotic death of dopamine cells. More specifically, dopaminegic neurons are shown to be susceptible to excitotoxicity induced by glutamate [615]. Cytokines control dopamine release indirectly via stimulation of glutamate release from astrocytes [233]. In *in vitro* studies conducted on mixed neuronal-glial primary cell cultures (microglia, astrocytes and cortical neurons) isolated from IL-1ra-/- and WT mice, basal and excitotoxins (NMDA and AMPA (promote Ca^{2+} influx into the cell)-induced cell death of cortical neurons in IL1-ra-/- mice was significantly higher than in the cell culture of WT mice [616]. In particular, it was demonstrated that IL-1ra secreted from microglia prevented the excitotoxicity in cortical cell culture isolated from WT mice [616]. Astrocytes have been shown to be the primary target for the action of IL-1 [617]. In addition, toxins such as rotenone (used in rodent model of Parkinson`s disease) and the herbicide paraquat are shown to increase vulnerability of dopamine neurons to excitotoxicity by potentiating cellular responses to glutamate neurotoxicity and by increasing Ca^{2+} influx [618, 619]. These observations imply that excess of IL-1β produced by activated microglia cells could have induced secretion of glutamate from astrocytes and promote additional excitotoxic

degeneration of dopamine neurons in IL-1ra-/- mice in absence of the neutralizing effect of IL-1ra secreted from the microglia.

Measured levels of MCP-1 in the plasma of IL-1ra-/- mice have indicated that single and repeated LPS injections had significantly reduced the level of MCP-1. Similar decrease in MCP-1 was observed in five LPS injected Casp-1-/- mice. For example, it was previously shown that partial hepatectomy in IL-1ra-/- mice increased MCP-1 level in the blood after four hours but a week later the levels of MCP-1 appeared to drop below the initial baseline despite an elevated plasma level of IL-1β [620]. It might be assumed that MCP-1 expression and secretion in blood was associated with some other metabolic alterations and was not dependent on IL-1 signaling. Therefore, future studies should be carried out in order to determine the regulation of MCP-1 secretion in the blood.

The absence of caspase-1 signaling significantly reduced the number of activated microglial cells (approx. 50%) and concomitantly this might have protected the dopamine neurons in Casp-1-/- mice against repeated LPS-induced degeneration. It was previously shown that IL-1 plays important role in the activation of microglia cells [621]. It has been demonstrated that Casp-1-/- mice had a decreased expression of CD68 in the liver in diet-induced steatohepatitis [622]. Moreover, caspase-11 -/- mice are demonstrated to be resistant against intranigral LPS-induced degeneration of TH-positive cells and had significantly less activated microglial cells in SNpc [623]. In particular, caspase-11 is reported to contribute to the secretion of IL-1β [624]. With regards to the signal transduction of LPS, both caspase-1 -/- and caspase-11 -/- mice are highly resilient to septic shock induced by live *Escherichia coli* or LPS [625, 626]. In conclusion, Casp-1-/- mice have been protected against repeated LPS systemic inflammation after fifteen months from the first saline/LPS injection.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on inflammatory outcomes.

- Long-term feeding on a high-fat diet coupled with repeated systemic LPS injections induced loss of dopamine neurons but it was not accompanied with increased number of activatated microglia cells. MCP-1 plasma levels were

similar to RD-fed mice and were not further exacerbated by single and repeated LPS injections.

There was no substantial evidence that long-term high-fat diet consumption exacerbated the loss of dopamine neurons in SNpc of WT mice. Repeated LPS injections had induced 11% loss of TH-positive cells in mice fed a high-fat diet, but this loss was not different from the total number of dopamine neurons that were quantified in five LPS-injected RD-fed mice. Surprisingly, HFD-fed mice had significantly less number of activated microglial cells in comparison to five times LPS-injected RD mice. As described in the introduction (chapter 5.1) microglia cells in HFD-fed mice could have additionally produced more proinflammatory cytokines such as TNF-α [602] that could have finally led to a similar degree of dopaminergic neurodegeneration in both HFD and RD-fed mice.

The plasma level of MCP-1 was not elevated or further affected by LPS treatment in HFD-fed mice. In that regard, the condition of excessive food intake and fat accumulation was shown to trigger hyperactive adipocytes to release MCP-1 that further stimulates infiltration of macrophages into the adipose tissue [332]. In parallel to the previous statement, the MCP-1 plasma level was shown to be elevated in two-month old mice that were fed a high-fat diet in a period of two months [351]. In the current study, HFD-fed mice tended to have higher accumulation of fat, but it did not result in a higher plasma level of MCP-1. As described before, adipose cell enlargement leads to the secretion of chemokines (e.g. MCP-1) [627], suggesting that MCP-1 could not have been elevated in HFD-fed mice since body weight gain in these 15 month-old mice was in stagnation.

Conclusions:

AIM 1: Long-term effect of systemic LPS challenge on inflammatory outcomes, exerted through the IL-1 pathway:

Major differences in inflammatory outcomes in the substantia nigra were observed among the three genotypes (WT, Casp-1-/- and IL-1ra-/-), whereas LPS effect was modest.

- Dysregulation in the IL-1 signaling pathway observed in IL-1ra-/- could have potentially contributed to the degeneration of dopamine neurons in SNpc.

Loss of dopamine neurons could not be solely attributed to activation of microglia cells.

- Low inflammatory state in Casp-1-/- contributed to the protection of dopamine neurons.

AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on inflammatory outcomes.

- Repeated systemic LPS inflammatory challenge induced a loss of dopamine neurons to a certain degree in RD and HFD-fed mice, which could not have been further exacerbated by low-grade chronic inflammation induced by high-fat diet consumption.

6. Discussion

A chronic, self-perpetuating neuroinflammatory process induced by activated microglial cells in the brain is associated with the progression of dopaminergic neurodegeneration [628]. A neuroinflammatory process may be elicited by various inflammatory challenges such as viruses and bacteria, environmental toxins, chronic inflammatory disorders (rheumatoid arthritis) and neuronal brain injuries [628-631]. Studies conducted in rodents suggest that chronic exposure to neurotoxins may advance dopaminergic neurodegeneration during aging [594]. In particular, dopamine neurons have been described to be relatively more susceptible to neuroinflammatory process than other neuronal subsets [238].

LPS stimulates the production of proinflammatory cytokines from local innate and adaptive immune cells [301] that further activate their own production by pericytes in the region of CVO`s and choroid plexus [62], endothelial cells of CVO`s [63] and BBB [64]. Moreover, LPS is also a potent stimulator of the TLR4 - NF-κB signaling pathway within the microglial cells, which elicits cytokine production via activation of caspase-1 [632]. The activation of the neuroinflammatory cascade, in particular the IL-1β and TNF-α pathways, are potent stimulators of microglial cells activation [633]. Microglia cells are mostly abundant in the region of substantia nigra; up to 12% of these cells were estimated to be within this brain region [237]. Activated microglial cells phagocyte injured dopaminergic neurons during the early phase of their apoptosis [634]. Moreover, microglia cells can facilitate the initiation of the programmed-cell death of dopaminergic neurons via proinflammatory cytokines (e.g. IL-1β and TNF-α) [260]. Damaged or dying dopaminergic neurons can also feedback positively the neuroinflammatory cascade driven by microglia cells by releasing neuromelanin, a byoproduct of catecholamine metabolism, which up-regulates the NF-κB signaling pathway in microglial cells that in turn produce proinflammatory cytokines [296]. Thus, this positive feedback loop develops a self-perpetuating neuroinflammatory process.

Aging is characterized by increased basal level of proinflammatory cytokines such as IL-1β, IL-6 and TNF-α in the periphery and the brain [301, 635]. Age-related priming of microglial cells is suggested to facilitate an environment that could be

described as a low-grade chronic neuroinflammation [636]. In a similar manner, high-fat diet consumption and obesity are also considered to facilitate a low-grade chronic inflammatory state [6] that could potentiate the up-regulation of peripherally-induced inflammation and exacerbate the loss of dopaminergic neurons. As it was previously shown, systemic LPS-induced inflammatory challenge caused a prolonged and delayed neurodegeneration of dopamine neurons [376]. Also, chronic low-grade central infusion of LPS caused prolonged neurodegeneration that was attributed to priming of microglia cells [476]. Therefore, it could be suggested that an acute high dose of systemic and/or low-grade chronic infusion of LPS may induce microglial cell priming and a self-perpetuating neuroinflammatory process that induces neurodegeneration of dopamine neurons, which progress over time.

6.1 AIM 1: Long-term effect of systemic LPS challenge on behavioral, metabolic and inflammatory outcomes, exerted through the IL-1 pathway.

In these studies, the data obtained from behavioral, metabolic and histological analysis suggested that the most important differences occurred among mice of the three different genotypes (WT, Casp-1-/- and IL-1ra-/- mice).

As presented in chapter 3, evaluation of motor activities in open-field test has shown that Casp-1-/- mice displayed increased; whereas, IL-1ra-/- mice showed initial hypoactivity and time-dependent decrease in locomotor activity. These data are in agreement with a previous study, where systemic administration of IL-1β suppressed locomotor activities in mice [478], whereas mice lacking IL-1R1-/- signalling displayed increased locomotor activities [479]. Overall, data are suggesting that motor activities are inversely related with IL-1β signalling pathway.

Over a course of nine months, IL-1ra-/- mice also displayed coordinative disabilities in the rotarod test. Decrease in both balance and motor activity could be attributed to the loss of dopamine neurons in SNpc and an extremely low level of leptin. It was suggested that leptin signaling in POMC neurons of severely obese, hypoactive and diabetic Lepr $^{db/db}$ mice increased locomotor activity [637]. Similarly, it has been demonstrated that juvenile *db/db* mice (5-6 weeks old) had lower locomotor activity in the open-field test than age-matched WT mice [638]. Our studies show that despite an increased overall locomotor activity within the open-field test, Casp-1-/- mice showed a trend of a time-dependent decrease in coordination in the rotarod test,

over a nine months period. We hypothesize that this mentioned trend in time-dependent decrease in coordination of Casp-1-/- mice could not be associated with dopamine depletion since these mice did not show signs of akinesia, a symptom that is usually associated with dopamine content deficiency [461]. This data might suggest that coordinative abilities in Casp-1-/- were partially protected. On the other hand, the saline-injected IL-1ra-/- mice displayed a profound decrease in motor coordination that reached significant differences versus saline-injected WT mice (figure 3.2). Interestingly, this difference was not exacerbated after the administration of LPS (figure 3.2). Moreover, the administration of LPS (1T and 5T) increased the variability in the experimental groups of the three genotypes and the significant differences among them were lost as shown in figure 3.2. Collectively, data suggest that during baseline conditions, an overall inflammatory state, due to the lack of IL-1ra, as observed in IL-1ra-/- mice, led to a major decrease in coordinative abilities. Thus, an IL-1β-induced neuroinflammatory process might have been involved in exacerbation of the coordinative disabilities observed in IL-1ra-/- mice in a time-dependent manner during baseline conditions.

In relation to non-motor symptoms, such as an anxiety/depressive-like behavior, Casp-1-/- mice displayed a tendency of anxiolytic behavior in elevated plus maze test; whereas, the IL-1ra-/- mice showed anxiety-like behavior and it progressed in time-dependent manner in the open-field test. In regards to Casp-1-/- mice, another mouse strain with the impairment in the IL-1β signaling pathway (IL-1R1-/- deficient mice), was shown to display anxiolytic behavior in the elevated plus maze test [479]. Thus, anxiety behavior in IL-1ra-/- mice could be attributed, at least in part to a neuroinflammatory process directed by IL-1β. Moreover, IL-1ra-/- mice display decreased adipogenesis [571] and evidence of decreased plasma levels of leptin [571]. As previously stated, that leptin treatment ameliorated anxiety behavior in *ob/ob* mice [639]; it can not be ruled out that low levels of leptin in IL-1ra-/- mice could also facilitate increased anxiety behavior. It is reported in this thesis that IL-1ra-/- mice showed a remarkable decrease of dopamine neurons as well. Since depletion of dopamine in medial prefrontal cortex of rats induced anxiety-like behavior, as observed in the elevated plus maze test [640]; therefore, it might also be possible that decreased dopamine neurons in IL-1ra-/- mice could also facilitate increased anxiety-like behavior. Collectively, the data of this thesis and literature

suggest that progression of anxiety-like behavior in IL-1ra-/- mice could be attributed to increased IL-1β-induced neuroinflammation, extremely low levels of leptin and loss of dopamine neurons.

Surprisingly, Casp-1-/- mice have appeared to develop anxiety-like behavior in the open-field test. However, this finding was not supported by another behavioral paradigm aimed at assessing anxiety behavior namely, the elevated plus maze test. Thus, it remains unclear if Casp-1-/- mice became more anxious in time-dependent manner and more studies should be carried out to further investigate this outcome. Anxiety behavior was described to be controlled by neuronal network that encompasses the hippocampal, cortical and afferents of the nucleus accumbens [641]. In particular, it has been demonstrated that dopamine action in dorsal hippocampus through its receptors (D1 and D2) modulates anxiety-like behavior [642]. Similarly, the functional interaction between NMDA and dopamine receptors in dorsal hippocampus has been recently associated with modulation of anxiety-like behavior [486]. In regard to a caspase-1 signaling, the inhibition of caspase-1 activity by the appropriate inhibitor (z-YVAD-FMK) has been shown to increase the NMDA receptor-mediated current in hippocampal slices *in vitro* [484, 485]. In connection with the previous statement, inhibition of NMDA signaling in dorsal hippocampus induced anxiolytic-like behavior in mice [486], suggesting time-related increase in activity of NMDA signaling in Casp-1-/- mice could have resulted in anxiety-like behavior in the open-field test. Overall, it could be suggested that increased IL-1β signaling might be involved in regulation of anxiety-like behavior in IL-1ra-/- mice. More studies are needed to clarify if Casp-1-/- mice become anxious in a time-dependent manner.

The prepulse inhibition test showed that pre-attentive function in Casp-1-/- mice was impaired, which could have possibly been associated with increased activity of D1 receptor [495] in the hippocampus as described in detail in the discussion of chapter 3. The prepulse inhibition test did not show any substantial cognitive impairment in IL-1ra-/- mice. These data suggest that cognitive function was not related to IL-1β signaling.

Final quantification of TH-positive neurons showed that dopamine neurons in Casp-1-/- mice were intact and their number tended to be even higher than in WT mice.

This was accompanied with very low activation of microglia cells. On the other hand, the loss of dopamine neurons in IL-1ra-/- mice could have been attributed to higher, but non-significant activation of microglial cells and overstimulation of IL-1 signaling, as described in the discussion of chapter 5. It was previously shown that IL-1 plays an important role in the activation of microglia cells [621]. Overall, these data suggest that IL-1β signaling was implicated in activation of microglia and subsequently played major role in dopaminergic neurodegeneration.

Surprisingly, single and repeated LPS inflammatory challenges had very modest or no effect on behavioral outcomes that were accompanied with very low loss of dopamine neurons. It is uncertain if the modest loss of dopamine neurons in LPS-injected WT mice may have been affected with LPS administration in mice at a very young age. In the current study, mice were injected when they reached 6 weeks of age, whereas in previous studies mice were injected after 10 weeks of age [376]. Basal inflammatory profile increases over time and it is uncertain if mice injected earlier with LPS could have recovered faster. In a previous study, 4-weeks old mice had better functional recovery and lower secretion of proinflammatory cytokines/chemokines by microglia than 10-weeks old mice in an experimental model describing the acute phase of spinal cord injury [643]. Therefore, it could be hypothesized that LPS injections at a younger age might have resulted in faster recovery and a decreased loss of dopamine neurons in WT mice.

Although LPS inflammatory challenge altered feeding behavior in WT and IL-1ra-/- mice and managed to induce decrease in BMD and BMC in WT and Casp-1-/- mice, other parameters such as glucose tolerance, insulin and leptin levels remained relatively unchanged. Overall, alteration in some of the metabolic parameters by LPS did not influence the behavioral outcomes, nor was it reflected on final count of dopamine neurons. However, repeated LPS treatments in WT mice increased the level of MCP-1 in plasma. It also appeared to a certain extent that long-term effects of LPS treatment caused a higher activation of microglia cells; however, this effect was only a trend that did not reach significant differences versus saline-injected mice. If this seemingly microglia activation occurred to a certain extent, this could have been associated with slight reduction of dopamine neurons observed in the WT mice treated with five monthly LPS injections. On the other hand, a significant decrease in MCP-1 levels of LPS-treated Casp-1-/- and IL-1ra-/- are not well

understood, but could possibly suggest that inflammatory actions in the periphery do not necessarily have to be associated with the inflammatory status in the brain. As it was previously demonstrated, MCP-1 and TNF-α were still elevated in the brain nine months after single systemic LPS injection [109]; whereas, TNF-α level in the blood was reduced to baseline [109]. As previously described in chapter 5, partial hepatectomy in IL-1ra-/- mice increased MCP-1 level in the blood after 4 hours [620]. A week later, the levels of MCP-1 tended to drop below the initial baseline despite an elevated plasma level of IL-1β [620]. It might be assumed that MCP-1 expression and secretion in blood were associated with some other metabolic alterations and were not dependent on IL-1 signaling. Therefore, future studies are needed to ascertain other possible regulators of MCP-1 secretion. Overall, Casp-1-/- mice did not show LPS-induced decrease of dopamine neurons and also had a very low number of activated microglia cells. However, the repeated LPS inflammatory challenge did not induce a further decrease of dopamine neurons in IL-1ra-/- mice nor it increased the number of activated microglia cells. It could be hypothesized that the loss of dopamine neurons in IL-1ra-/- mice could not be further exacerbated by the LPS inflammatory challenge. Since IL-1ra-/- mice are known to suffer from postnatal growth retardation [582], it might be hypothesized that neurogenesis of dopamine neurons may be impaired. In the future studies, time-dependent quantification of TH-positive cells in IL-1ra-/-mice should be carried out in order to better understand if the loss of dopamine neurons in IL-1ra-/- mice increases as they age.

6.2 AIM 2: Long-term effects of high-fat diet consumption and systemic LPS challenge on behavioral, metabolic and inflammatory outcomes.

High-fat diet feeding in mice did not result in impaired locomotor activity, which was confirmed by unaltered number of TH-positive cells in SNpc. Moreover, high-fat diet feeding did not result in increased number of activated microglial cells; however, previous study demonstrated that microglial cells in HFD-fed mice produced more TNF-α [602]. If the inflammatory cascade was exacerbated in the brain of HFD-fed mice, it has not resulted in the loss of dopamine neurons. On the other hand, repeated LPS inflammatory challenge induced 11% loss of dopamine neurons in HFD-fed mice, but this loss was not reflected in decreased locomotor activity in the open-field test. It is possible that there might be a certain threshold in

order for the loss of dopaminergic neurons to be reflected as decreased locomotor activity. For instance, it was previously shown that a significant loss of 37% of dopamine neurons caused decreased locomotor activity in the open-field test [376]. Therefore, it might be suggested that the small 11% loss of dopamine neurons could not have induced significant locomotor disability in 5-times LPS-injected HFD-fed mice. However, both saline and 5-times LPS-injected HFD-fed mice displayed similar time-dependent coordinative decrease in the rotarod test. Time-dependent decrease in coordinative ability could be associated with decreased activity of D2 receptor, which could have been induced by insulin and leptin insensitivity [368], and lower synthesis of TH enzyme due to the leptin resistance [366]. Collectively, impaired dopaminergic neurotransmission in HFD-fed mice could have resulted in reduced coordinative skills in the rotarod test. High-fat diet-fed mice have also shown symptoms of bradykinesia, akinesia and dyskinesia. It has to be considered that changes in the rotarod test caused by HFD do not seem to occur as a consequence of body weight gain. In fact, body weight of HFD-fed mice was similar to that of RD-fed mice. Therefore, it seems that motor impairments observed in HFD-fed mice (bradykinesia, akinesia and dyskinesia) may not be attributed to general body gain. Although these motor symptoms could have been associated with the loss of dopamine neurons, HFD-fed mice did not respond to the L-DOPA test in a similar manner as RD-fed mice did. For example, akinesia has been pharmacologicaly-induced in mice by inhibition of D2 receptors [499], and bradykinesia and dyskinesia were observed in DAT -/- mice [498, 501]. Since high-fat diet consumption and obesity in mice were associated with reduced activity of DAT [471] and decreased density of D2 receptors [500], these factors could have collectively affected motor activities in HFD-fed mice, with intact dopamine neurons.

Anxiety behavior in HFD-fed mice has been associated with insulin resistance as mice that develop spontaneous diabetes have displayed anxiety-like behavior in the elevated plus maze test [644]. In the current study, high-fat diet-fed mice did not show to be anxious in the open-field and elevated plus maze tests. However, significant anxiogenic behavior was observed in the novelty suppressed feeding test that might have been driven by overnight fasting, as described in the discussion of

chapter 3. On the other hand, LPS inflammatory challenge did not exacerbate the anxiety behavior of HFD-fed mice.

HFD-fed mice demonstrated impaired cognitive function in the prepulse inhibition test and displayed a trend to have impaired memory function in the Morris water maze test. Cognitive impairment could be attributed to low activity of DAT in HFD-fed mice as it was previously shown that DAT -/- mice had disrupted prepulse startle response [380]. Similarly, an age-dependent decrease in prepulse response was observed in *db/db* mice [638], suggesting a possible role of type-2 diabetes and leptin signaling dysfunction in cognitive processing. As it was previously described in the discussion of chapter 3, the desensitization of protein kinase B (Akt) signaling pathway that is coupled to leptin receptors in the hippocampus of high-fat diet-fed mice induced spatial memory impairment [513]. A cognitive decline in high-fat diet-fed mice was also attributed to the desensitization of NMDA receptors in the hippocampus and it was also observed that this process was compatible with the development of leptin resistance [514]. Similarly, insulin resistance in high-fat diet-fed mice decreased the release of dopamine and its clearance from synaptic clefts [12]. As it was reported that insulin resistance contributed to cognitive decline [448], this might explain a possible connection between dopamine signaling and insulin resistance-induced cognitive impairment in the prepulse inhibition test. These observations suggest that high-fat diet consumption induced cognitive and partially memory impairment through the altered neurotransmission in the hippocampus, which could have been related with leptin and insulin resistance.

Overall, feeding mice a high-fat diet induced metabolic perturbations that were related with some of the behavioral alterations that were described above. The reduced number of TH-positive neurons with repeated LPS injections did not affect the behavioral outcomes. High-fat diet consumption did not exacerbate the loss of dopamine neurons under LPS inflammatory challenge in comparison to RD-fed wild type mice. The loss of dopamine neurons with repeated LPS injections could not be completely associated with relatively similar amount of CD68-positive microglia nor MCP-1 level; therefore, additional in depth experiments are needed to ascertain the inflammatory profile in LPS-injected HFD-fed mice.

In conclusion, on one hand, the data reported in this thesis suggest that dysregulation of the IL-1 signaling pathway observed in IL-1ra-/- mice could have potentially contributed to the delayed degeneration of dopamine neurons in SNpc. This was observed by decreased locomotor and coordinative abilities. On the other hand, low inflammatory state in Casp-1-/- mice contributed to the protection of dopamine neurons and preserved motor activities; however, other behavioral outcomes could have been attributed to the altered dopamine-mediated neurotransmission. A long-term effect of LPS inflammatory challenges tended to induce the dopamine neurodegeneration in RD-fed wild type mice. Although, repeated LPS injections induced a small depletion of dopaminergic neurons in HFD-fed mice, this loss was not further exacerbated by a low-grade chronic inflammation induced by high-fat diet consumption.

6.3 Future directions

In order to better understand the role of glutaminergic and dopamine-mediated neurotransmission and its modulation by metabolic disorders on behavioral outcomes, further studies are required at the level of dopamine receptors such as D1, D2 and the dopamine transporter (DAT). Extensive inflammatory profiling in the periphery and the brain would be needed in IL-1ra-/-, Casp-1-/- and HFD-fed mice. Initial baseline quantification of TH-positive neurons in IL-1ra-/- mice would be valuable in order to determine possible differences among the three genotypes. In future studies, systemic LPS inflammatory challenge in older mice should be ascertained as this might elicit more profound effects on dopaminergic neurodegeneration. Since anti-inflammatory drugs such as anakinra ameliorated the neuroinflammatory process and toxin-induced degeneration of dopamine neurons in rodents [158], it could be proposed that nigrostriatal administration of IL-1ra in IL-1ra-/- mice would down-regulate the neurodegenerative process in the substantia nigra. In order to explore and emphasize the importance of the IL-1 pathway in degeneration of dopamine neurons, effects of nigrostriatal administration of IL-1β in Casp-1-/- mice could be tested and determined in future studies.

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